Remarks

Remarks Regarding Claim Amendments

The amendment to claim 1 is supported by canceled claims 13-16. The amendments to claims 10 and 17 are supported by original claims 10 and 17.

No new matter is added and the entry of the claim amendments is requested.

Remarks Regarding 35 USC §112 First Paragraph

Claims 1-24 stand rejected under 35 U.S.C. 112 first paragraph as allegedly non-enabling. Applicants traverse.

Applicants' claimed invention, as reflected in amended claim 1, is directed to a method of affecting the differentiation of a neural stem cell or neural progenitor cell into neurons. In the method, a neural stem cell or neural progenitor cell in a sample is contacted with a Groucho-interacting protein (GIP) to allow for the formation of a GIP-Groucho corepressor complex. This complex represses DNA transcription and by suppressing alternative pathways of differentiations, guides the cell into differentiating into a neuron (See, claim 1, page 4, lines 14-20). By using this method, the specification teaches that cells can be differentiated into motor neurons cells (page 4, lines 30-31), interneuron, projection neurons (including dopaminergic neuron, cortical neuron, gabaergic neuron, glutaminergic neuron).

The specification has provided ample support and description on all aspects of the claimed method. Various aspects of the claimed method, as well as modes of operation, are described in detail. For example, the choice of appropriate GIP and Gro/TLE corepressors are shown on page 77. On the same page, Applicants have shown that these GIPs may be applied to cells to reduce transcription and gene expression levels. This repression activity is increased, up to 10 folds, in the presence of co-repressors (page 78, lines 9-13). Applicants have provided disclosures showing how GIPs can mediate neural patterning activities (page 80 to page 81, line 2) and that the ectopic expression of GIPs respecify enuronal cell fate (page 81, lines10-12).

Additional disclosures showing how GIPs may be used to cause the expression of ectopic V3 neurons (page 82 first three paragraphs) are also provided.

Furthermore, the Examiner alleges that Nkx2.2 is capable of binding any other Groucho-corepressor proteins or that Grg4 is capable of binding any other GIP. Applicants respectfully traverse.

It is well-established that the vertebrate Groucho homologues, Grg1-4, are structurally and functionally related (see, e.g., Eberhard et al., 2000, *EMBO J.*, 19:2292-2303; Exhibit 1, pages 2299-2300). Published experiments have shown that that the *Drosophila* Groucho protein shares overlapping functions with Groucho co-repressors in higher vertebrates (Exhibit 1, pages 2299-2300). Thus, one of skill in the art would know that there is significant evidence that Grg1, Grg2, Grg3 or Grg4 would have similar functions and that there is no reason to believe that Grg1, Grg2, Grg3 and Grg4 cannot be used in combination or interchangably with one another. In fact, Grg1, Grg2, Grg3 and Grg4 are expressed together in naturally occurring cellular systems.

Further, Nkx2.2 and Nkx2.9 share closely related structures, expression patterns, and dependence on Shh during early stages in embryogenesis (see, e.g.., Pabst et al., 2000, *Devel. Genes Evol.* 210:47-50; Exhibit 2, pages 48-49). In addition, available data indicate that Nkx6.1 and Nkx6.2 exhibit closely related structures and overlapping functions, and only double mutants of Nkx6.1 and Nkx6.2 show complete loss of Nkx6 activity (see, e.g., Vallstedt et al., 2001, *Neuron* 31:743-755; Exhibit 3, page 748). This would indicate to one of skill in the art that these two proteins would have overlapping functions.

In view of Applicants' arguments, it is asserted that the instant application has met the requirements for enablement under 35 U.S.C. §112. Withdrawal of the rejection of claims 1-24 is respectfully requested.

Remarks Regarding 35 USC §112 Second Paragraph

Claims 1-24 stand rejected under 35 U.S.C. 112 second paragraph as allegedly indefinite. Applicants traverse.

Claim 1 is allegedly indefinite because the phrase "the cell" in line 3 lacks antecedent basis. Applicants have amended the claim to recite "the neural stem cell" and antecedent basis for this term is provided in the preamble (line 1) of the claim 1.

Claim 1 is allegedly indefinite because of the terms "a specific cell," and "alternative pathways of differentiation." These terms have been removed.

Claims 2-24 are allegedly indefinite because they depend on claim 1. Since claim 1 has been amended and is no longer indefinite, the rejection of claims 2-24 is now moot.

Based on Applicants' amendments, the rejection of claims 1-24 under 35 U.S.C. 112 second paragraph is moot and should be withdrawn.

CONCLUSION

On the basis of the foregoing amendment and remarks, Applicant respectfully submits, that the pending claims are in condition for allowance. If there are any questions regarding these amendments and remarks, the Examiner is encouraged to contact the undersigned at the telephone number provided below.

If the enclosed papers are considered incomplete, the Mail Room and/or the Application Branch is respectfully requested to contact the undersigned at 212-935-3000, New York, New York.

The Director is authorized to charge all fees that may be due, or to credit any overpayment, to the undersigned's account, Deposit Account No. **50-0311**, Ref. No. **21882-502**, Customer No. **35437**.

Respectfully submitted,

Date: October 20, 2005

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The EMBO Journal Vol. 19 No. 10 pp. 2292–. , 2000

Transcriptional repression by Pax5 (BSAP) through interaction with corepressors of the Groucho family

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Pax5 (BSAP) functions as both a transcriptional activator and repressor during midbrain patterning, B-cell development and lymphomagenesis. Here we demonstrate that Pax5 exerts its repression function by recruiting members of the Groucho corepressor family. In a yeast two-hybrid screen, the *groucho*-related gene product Grg4 was identified as a Pax5 partner protein. Both proteins interact cooperatively via two separate domains: the N-terminal Q and central SP regions of Grg4, and the octapeptide motif and C-terminal transactivation domain of Pax5. The phosphorylation state of Grg4 is altered *in vivo* upon Pax5 binding. Moreover, Grg4 efficiently represses the transcriptional activity of Pax5 in an octapeptide-dependent manner. Similar protein interactions resulting in transcriptional repression were also observed between distantly related members of both the Pax2/5/8 and Groucho protein families. In agreement with this evolutionary conservation, the octapeptide motif of Pax proteins functions as a Groucho-dependent repression domain in *Drosophila* embryos. These data indicate that Pax proteins can be converted from transcriptional activators to repressors through interaction with corepressors of the Groucho protein family.

Keywords: Groucho (Grg)/interaction partners/Pax5 (BSAP)/transcriptional repression

Introduction

The Pax proteins constitute a family of paired domain-containing transcription factors that play essential roles in early development from *Drosophila* to man. Based on sequence similarities, these *Pax* genes can be grouped into four subfamilies (Noll, 1993). One of them consists of a single *Drosophila* member, *dPax258* (*spa*) (Czerny *et al.*, 1997; Fu and Noll, 1997), and three mammalian genes, *Pax2*, *Pax5* and *Pax8*, which arose by gene duplications at the onset of vertebrate evolution (Pfeffer *et al.*, 1998).

Pax5 codes for the transcription factor BSAP, which is essential for brain patterning and B-lymphopoiesis (reviewed by Busslinger and Nutt, 1998). *Pax5* cooperates with *Pax2* in development of the midbrain and cerebellum, in agreement with the overlapping expression patterns of the two genes at the midbrain–hindbrain boundary of the mouse embryo (Urbánek *et al.*, 1997). In contrast, *Pax5* is the only member of the *Pax* family that is expressed in B-lymphocytes (Adams *et al.*, 1992). As a consequence, B-cell development is arrested at an early pro-B cell stage in mice lacking *Pax5* (Urbánek *et al.*, 1994). Surprisingly, the Pax5-deficient pro-B cells are not yet restricted in their lineage fate. Instead, these cells are able to differentiate into various myeloid and lymphoid cell types *in vitro* as well as *in vivo* and thus retain a broad developmental potential characteristic of an uncommitted hematopoietic progenitor cell (Nutt *et al.*, 1999; Rolink *et al.*, 1999). Pax5-deficient pro-B cells are, however, able to develop along the B-lymphoid lineage once *Pax5* expression has been restored by retroviral transduction. These experiments therefore identified Pax5 as the critical B-lineage commitment factor that restricts the developmental potential of progenitor cells to the B-lymphoid pathway by suppressing alternative cell fates (Nutt *et al.*, 1999; Rolink *et al.*, 1999).

Insight into the transcriptional role of Pax5 has been provided by the identification of target genes, which was facilitated by the *in vitro* culture of Pax5-deficient pro-B cells and the development of a Pax5-specific induction system (Nutt *et al.*, 1998). Pax5 was thus shown to activate *CD19*, *Igα* (*mb-1*), *LEF-1* and N-*myc* expression and simultaneously to repress *PD-1* transcription (Nutt *et al.*, 1998). Pax5 therefore fulfills a dual role in early B-cell development, as it functions as both an activator and repressor of gene transcription. The repression function of Pax5 is particularly important for the suppression of alternative lineage fates at B-lineage commitment, which is best illustrated by the regulation of the *M-CSF-R* gene. This myeloid gene is one of several hematopoietic genes that are expressed promiscuously in the uncommitted Pax5-deficient pro-B cell. Upon commitment, Pax5 represses *M-CSF-R* transcription, thus rendering B-cell precursors unresponsive to the myeloid cytokine M-CSF (Nutt *et al.*, 1999). Furthermore, Pax5 has been implicated in the repression of the J-chain gene and down-regulation of the activity of immunoglobulin 3′ enhancers during late B-cell differentiation (reviewed by Busslinger and Nutt, 1998).

Structure–function analyses are also consistent with a dual role for Pax5 in transcriptional regulation (see Figure 1A). Pax5 is known to recognize target genes via its N-terminal paired domain and to control transcription through a C-terminal regulatory module consisting of activating and inhibitory sequences (Dörfler and Busslinger, 1996). Furthermore, Pax5 possesses a characteristic octapeptide that was identified originally as a conserved sequence motif found in most Pax proteins (Burri *et al.*, 1989; Noll, 1993). The presence of this octapeptide motif was subsequently shown to down-modulate the transcriptional activity of Pax proteins

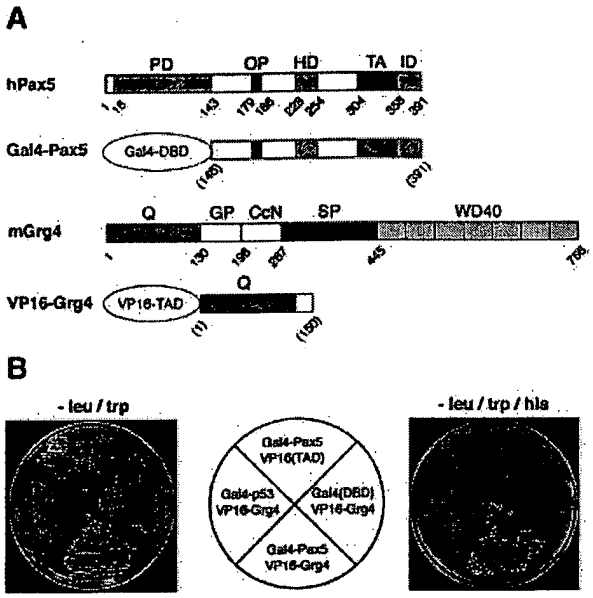


Fig. 1. Interaction of Pax5 with the N-terminal region of Grg4 in yeast. (A) Schematic diagram of Pax5, Grg4 and the respective fusion proteins. The different domains of each protein are indicated together with the corresponding amino acid positions. The chimeric Gal4–Pax5 protein was used as a bait to isolate the VP16-Grg4 fusion protein in a yeast two-hybrid screen of a cDNA expression library that was generated by fusing the transactivation domain (TAD) of VP16 to cDNA derived from 9.5/10.5-day-old mouse embryos (Hollenberg *et al.*, 1995). See text for description of the Grg4 structure. PD, paired domain; OP, octapeptide; HD, partial homeodomain; TA, transactivation region; ID, inhibitory domain; DBD, DNA-binding domain. (B) Specific interaction of Pax5 and Grg4 in yeast. Expression plasmids of the combinations indicated (central panel) were transformed into yeast and selected for by growth on plates lacking leucine and tryptophan (left panel). Activation of a Gal4-dependent *HIS3* gene was examined by growth on selection plates additionally lacking histidine (right panel).

(Lechner and Dressler, 1996). Pax5, like other members of the Pax2/5/8 family, also contains a partial homeodomain that constitutes an interaction surface for both the retinoblastoma (Rb) gene product and the TATA-binding protein (Eberhard and Busslinger, 1999).

As the function of DNA-binding transcription factors is determined by the interaction with cofactors, we have employed the yeast two-hybrid assay to search systematically for Pax5 partner proteins. Here, we describe the identification and characterization of Grg4 as a Pax5 interaction partner. Grg4, which is also known as TLE4, is one of four members of the mammalian Groucho family (Stifani *et al.*, 1992; Koop *et al.*, 1996). The founding member of this conserved protein family is the *Drosophila* Groucho protein, which is broadly expressed throughout development and plays important roles in diverse processes such as sex determination, segmentation and neurogenesis (Paroush *et al.*, 1994). The Groucho proteins consist of several conserved domains including the N-terminal glutamine-rich Q region and C-terminal WD40 repeats (see Figure 1A). Although the Groucho proteins are localized in the nucleus, they lack any recognizable DNA-binding motif. Instead, these proteins interact with various DNA-binding transcription factors and are thus recruited to specific control regions where they function as potent corepressors to inhibit gene transcription (reviewed by Fisher and Caudy, 1998; Parkhurst, 1998). The Groucho proteins form higher order complexes by tetramerizing via the Q domain (Chen *et al.*, 1998), bind to the N-terminal tails of histone H3 (Palaparti *et al.*, 1997), interact with histone deacetylases (Chen *et al.*, 1999; Choi *et al.*, 1999) and thus seem to exert their function as part of multiprotein–DNA complexes that locally establish a repressive chromatin structure.

Here we demonstrate by protein binding and co-immunoprecipitation assays that Pax5 is able to interact with the Grg4 protein *in vitro* as well as *in vivo*. Both proteins contact each other via two separate interaction domains. The N-terminal Q domain and central SP region of Grg4 interact cooperatively with the C-terminal transactivation domain and octapeptide motif of Pax5, respectively. Moreover, the interaction with Pax5 induces a specific change in the phosphorylation state of Grg4. As shown by transient transfection assays, Grg4 can repress the transcriptional activity of Pax5 efficiently in an octapeptide-dependent manner. Similar protein interactions resulting in repression were also observed between distantly related members of the Pax2/5/8 and Groucho protein families. As predicted by this evolutionary conservation, the octapeptide motif was shown to function as a Groucho-dependent repression domain in *Drosophila* embryos. Together, these data indicate that Pax proteins can function as active repressors by recruiting corepressors of the Groucho family to selected target genes, thus offering a molecular explanation of how Pax5 represses the transcription of non-B-lymphoid genes at B-lineage commitment.

Results

Identification of Grg4 as an interaction partner of Pax5

We employed the yeast two-hybrid system to search for potential cofactors of Pax5. To this end, a Gal4–Pax5 fusion protein (Figure 1A) was used as a bait to screen a VP16-tagged cDNA expression library of mouse mid-gestation embryos. One of the isolated cDNA clones encoded a polypeptide that interacted specifically with Pax5 but not with a Gal4–p53 protein or with the Gal4 DNA-binding domain alone (Figure 1B). cDNA sequence analysis revealed that the VP16 transactivation domain of the expression vector was fused in-frame to a 150 amino acid polypeptide that is most highly related to the N-terminal sequences of the *Xenopus* and rat Grg4 (Esp2) proteins, two vertebrate homologs of *Drosophila* Groucho (Schmidt and Sladek, 1993; Roose *et al.*, 1998). As only a partial sequence of the mouse Grg4 protein has been characterized thus far (Koop *et al.*, 1996), we cloned the full-length murine *Grg4* cDNA by RT–PCR. Like other members of the Groucho family, the mouse Grg4 protein consists of five characteristic domains (Figure 1A): a highly conserved glutamine-rich Q domain at the N-terminus; the GP domain enriched in glycine and proline residues; the conserved CcN domain containing a nuclear localization signal and putative phosphorylation sites for casein kinase II and cdc2 kinase; a serine/proline-

rich region referred to as the SP domain; and a C-terminal region containing seven highly conserved WD40 repeats (Stifani *et al.*, 1992; see note added in proof). Interestingly, the VP16-Grg4 protein isolated in the yeast two-hybrid screen contains the entire Q region and part of the GP domain (Figure 1A), indicating that these N-terminal sequences of Grg4 can interact specifically with Pax5 in the yeast cell.

The transactivation domain of Pax5 interacts with the Q domain of Grg4

To verify the Pax5–Grg4 protein interaction in murine cells, we next performed one-hybrid assays in transiently transfected J558L plasmacytoma cells that do not express endogenous Pax5. The expression of limiting amounts of Pax5 protein resulted in a modest increase of the transcriptional activity of the luciferase gene *luc-CD19* (Figure 3A), which is under the control of three high-affinity Pax5-binding sites (Dörfler and Busslinger, 1996). Co-expression of the VP16-Grg4 polypeptide, which was isolated in the yeast two-hybrid screen, enhanced the luciferase activity ~5-fold, whereas the VP16 transactivation domain alone had no effect. Hence, this potent transactivation domain is recruited to the promoter only when linked to the N-terminal Grg4 sequences. Moreover, this recruitment depends on Pax5, as in its absence the VP16-Grg4 protein failed to stimulate the basal promoter activity (Figure 3A). We conclude, therefore, that the Pax5 protein and the N-terminal domain of Grg4 interact with each other in mammalian cells, thus confirming the results of the yeast two-hybrid assay. Importantly, a VP16-Gro protein containing the equivalent N-terminal sequences of *Drosophila* Groucho enhanced the activity of the reporter gene to the same level as VP16-Grg4 (Figure 3A), indicating that the Pax5 interaction domain has been conserved among Groucho proteins.

We next took advantage of the same one-hybrid assay to delineate the domain of Pax5 that is required for interaction with the N-terminal Q domain of Grg4. For this purpose, we analyzed a series of mutant Pax5 proteins that are shown schematically in Figure 2A. The C-terminal sequences of Pax5 are known to harbor a potent transactivation (TA) domain that is negatively regulated by adjacent inhibitory sequences (Dörfler and Busslinger, 1996). Deletion of this inhibitory domain in the mutant protein B4 did not interfere with the Pax5-dependent stimulatory effect of VP16-Grg4 (Figure 3B). In contrast, transcriptional stimulation was abolished by further deletion of the C-terminal transactivation domain in the Pax5 mutants B8 and B9, whereas internal deletion of the conserved octapeptide motif (ΔOP) or the partial homeodomain (ΔHD) of Pax5 did not have any effect (Figure 3B). These results indicate that the C-terminal transactivation domain of Pax5 interacts specifically with the N-terminal Q domain of Grg4.

The interaction of Pax5 with full-length Grg4 depends on the Pax5 octapeptide motif and the Grg4 SP domain

We next used *in vitro* protein-binding assays to study the interaction between Pax5 and Grg4. To this end, we expressed a protein consisting of the Q domain of Grg4 fused to glutathione *S*-transferase (GST) (Figure 4A) for

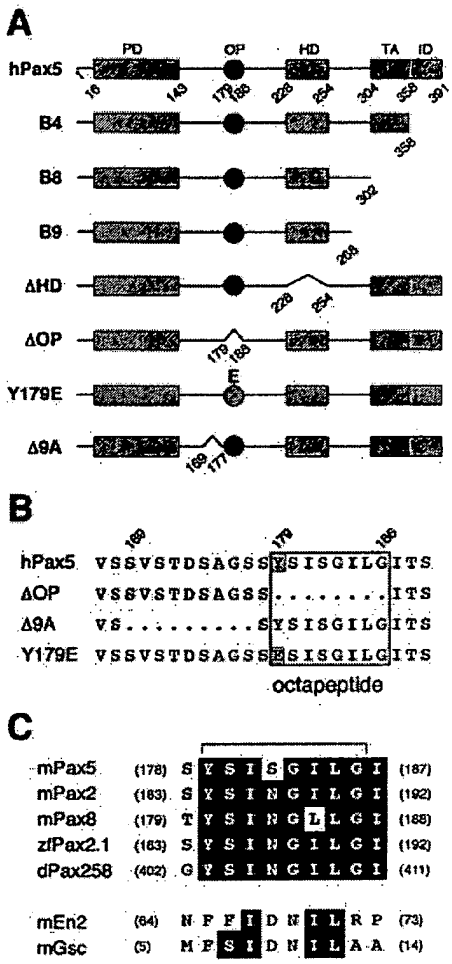


Fig. 2. Schematic diagram of mutant Pax5 proteins. (**A**) Domain structure of Pax5 and extent of amino acid deletion in the different mutant proteins. (**B**) The octapeptide sequence is shown together with the Y179E mutation and the ΔOP and Δ9A deletions. (**C**) Conservation of the octapeptide motif. The octapeptide sequences of mouse (m), zebrafish (zf) and *Drosophila* (d) members of the Pax2/5/8 family are aligned with the corresponding Engrailed homology region 1 (eh1) of the homeodomain transcription factors En2 and Goosecoid (Gsc). Identical amino acids are highlighted by black overlay. For sequences, see Smith and Jaynes (1996), Czerny *et al.* (1997) and Pfeffer *et al.* (1998).

subsequent use in GST pull-down assays. Surprisingly, this Grg4-Q polypeptide, like the control GST protein, failed to bind radiolabeled Pax5 protein (Figure 4B, lanes 2 and 7). In contrast, a GST fusion protein containing the entire Grg4 sequence interacted efficiently with Pax5 (Figure 4B, lane 3), indicating that full-length Grg4 and Pax5 can form a relatively stable complex *in vitro* and that Grg4 sequences other than the N-terminal Q domain are also involved in complex formation. To identify this additional domain(s), we analyzed a series of GST fusion proteins with progressively larger deletions of C-terminal Grg4 sequences (Figure 4A). Elimination of the WD40 repeats in the mutant Grg4-ΔWD40 protein did not significantly affect the interaction with Pax5 (Figure 4B, lane 4), indicating that these protein–protein interaction

motifs (Fisher and Caudy, 1998) are not essential for Pax5 binding. However, further deletion of the SP domain (Grg4-ΔSP) resulted in a dramatic reduction of Pax5 binding, thus identifying this central region of Grg4 as a second Pax5 interaction domain. Interestingly, a GST fusion protein containing only the SP domain was unable to interact with Pax5 (data not shown), suggesting that Pax5 binding depends on cooperative interaction with both the SP and Q domains of Grg4.

The GST pull-down experiments also pointed to the existence of an additional Grg4-binding region in Pax5. To identify this domain, we analyzed mutant Pax5 proteins (Figure 2A) for their ability to bind full-length Grg4 in the GST pull-down assay (Figure 4C). The deletion mutant B8 bound Grg4 with an affinity similar to the full-length Pax5 protein, indicating that this assay failed to detect an interaction between the C-terminal transactivation domain of Pax5 and the N-terminal Q domain of Grg4, in agreement with the data shown in Figure 4B (lane 7). Grg4 binding was, however, dramatically reduced by deletion of the octapeptide motif (ΔOP) in Pax5, whereas elimination of nine amino acids (Δ9A) adjacent to the octapeptide sequence did not influence protein binding (Figure 4C). The octapeptide motif of Pax2/5/8 proteins is closely related to a short sequence present in the repression domain of the transcription factors Engrailed (En) and Goosecoid (Gsc; Figure 2C). This short domain is known as En homology region 1 (eh1) or Gsc-En homology (GEH) element (Smith and Jaynes, 1996) and was previously shown to mediate interaction with the *Drosophila* Groucho protein (Jiménez *et al.*, 1997, 1999). Groucho binding was, however, abolished by mutating a conserved phenylalanine of the eh1/GEH sequence to glutamic acid (Jiménez *et al.*, 1999). The octapeptide sequence of all known Pax2/5/8 proteins contains a tyrosine residue at the corresponding position (Figure 2C), which could, however, be substituted by phenylalanine without affecting Grg4 binding (data not shown). In contrast, the glutamic acid substitution Y179E completely abrogated the interaction of Pax5 with Grg4 (Figure 4C), further emphasizing the importance of the octapeptide motif as an interaction domain for Grg4.

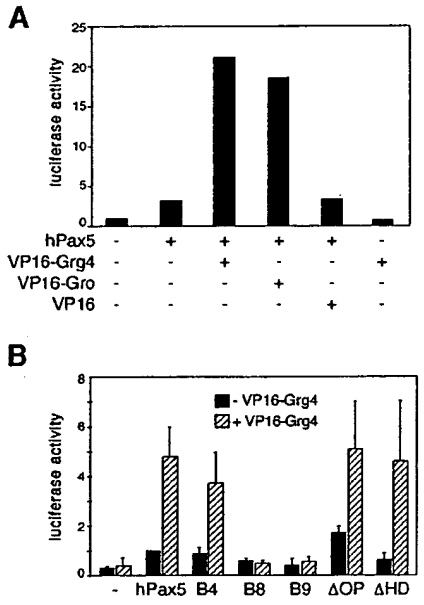


Fig. 3. The N-terminal region of Grg4 interacts with the transactivation domain of Pax5. (**A**) Interaction of Pax5 and VP16-Grg4. The expression plasmids indicated (100 ng) were co-transfected into J558L cells together with the *Renilla* luciferase gene pRL-SV40 (0.4 μg) and firefly luciferase gene *luc-CD19* (5 μg; Dörfler and Busslinger, 1996). After 48 h, the cells were lysed, luciferase activities were measured, and the activity of the firefly luciferase was standardized relative to the control *Renilla* luciferase to normalize for differences in transfection efficiencies. Luciferase values are shown relative to the activity measured with the empty expression vector pKW2T (left bar). (**B**) Interaction of the Q domain of Grg4 with the transactivation domain of Pax5. Both luciferase genes and the Pax5 expression plasmids indicated (100 ng each) were electroporated into J558L cells with (hatched bars) or without (black bars) the VP16-Grg4 expression vector (100 ng). Average values of three experiments are shown.

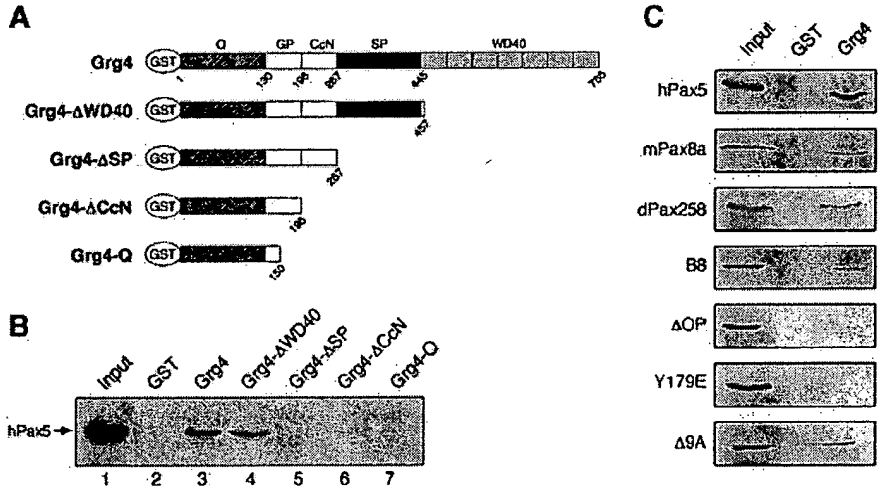


Fig. 4. *In vitro* binding of Pax5 to Grg4. (**A**) C-terminal deletions of GST–Grg4 proteins. (**B**) The SP domain of Grg4 interacts with Pax5. GST pull-down assays were used to study the interaction between *in vitro* translated, ^{35}S-labeled Pax5 protein and GST (lane 2) or GST–Grg4 proteins (lanes 3–7) bound to glutathione–Sepharose. Lane 1 contained 10% of the Pax5 protein input. (**C**) The octapeptide of Pax5 mediates binding of Grg4. The ^{35}S-labeled Pax proteins indicated were analyzed for binding to GST or GST–Grg4. The input lane contained ~10% of the total Pax protein used in each assay.

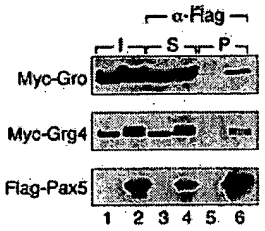


Fig. 5. *In vivo* interaction of Groucho and Pax5. Myc-tagged Grg4 or Groucho proteins were expressed in transiently transfected COP-8 fibroblasts either alone (lanes 1, 3 and 5) or with a Flag-tagged Pax5 protein (lanes 2, 4 and 6). After 48 h, whole-cell lysates were prepared and immunoprecipitated with the anti-Flag M2 antibody. Immunocomplexes were analyzed by Western blotting first with a polyclonal anti-Myc antibody and then with a polyclonal antiserum directed against the Pax5 paired domain (Adams *et al.*, 1992). Lanes 1–4 contained ~7.5% of the total input (I) protein or supernatant (S), respectively. P, precipitated protein.

In vivo interaction of Grg4 and Pax5

To investigate complex formation *in vivo*, we expressed Myc epitope-tagged Grg4 or Groucho either alone or together with Flag-tagged Pax5 in transiently transfected COP-8 fibroblasts. Pax5 was precipitated subsequently from the cell lysate with a monoclonal anti-Flag antibody, and the immunoprecipitate was analyzed for the presence of Groucho and Pax5 proteins by Western blotting. A low but significant amount of Myc-tagged Grg4 or Groucho was only detected in the immunoprecipitate of COP-8 cells co-expressing Pax5 (Figure 5, compare lanes 5 and 6). These data therefore indicate that the mouse Grg4 and *Drosophila* Groucho proteins can interact with Pax5 *in vivo*. However, we failed to co-immunoprecipitate endogenous Pax5 and Grg proteins from nuclear extract of B cells, which may reflect a low abundance or stability of the Pax5–Grg complex (see Discussion).

Induced phosphorylation of Grg4 upon interaction with Pax5

The *Drosophila* Groucho and mammalian Grg proteins are expressed predominantly as a constitutively phosphorylated polypeptide that can be detected as a single band of ~90 kDa by Western blot analysis (Husain *et al.*, 1996). Additional phosphorylation of Grg proteins was observed upon neural differentiation of P19 embryonal carcinoma cells, which resulted in the appearance of a doublet of 90–93 kDa (Husain *et al.*, 1996). Similar Grg isoforms were also generated in COP-8 cells co-expressing Grg4 and Pax5 (Figure 5, lanes 2 and 4). However, a single Grg4 species was detected in the absence of Pax5 (Figure 5, lanes 1 and 3), suggesting that the interaction with Pax5 leads to further modification of the Grg4 protein. Moreover, both Grg4 isoforms could be co-immunoprecipitated together with Pax5 from COP-8 cell extracts (Figure 5, lane 6). Incubation of the immunoprecipitate with λ protein phosphatase resulted in a single Grg4 species that migrated slightly faster on SDS–PAGE than both isoforms prior to phosphatase treatment (Figure 6A). Hence, we conclude that the two Grg4 isoforms expressed in COP-8 cells differ in their phosphorylation state.

The Pax5-dependent phosphorylation of Grg4 suggested that binding of Grg4 to Pax5 is a prerequisite for this modification to occur. To test this hypothesis, we co-

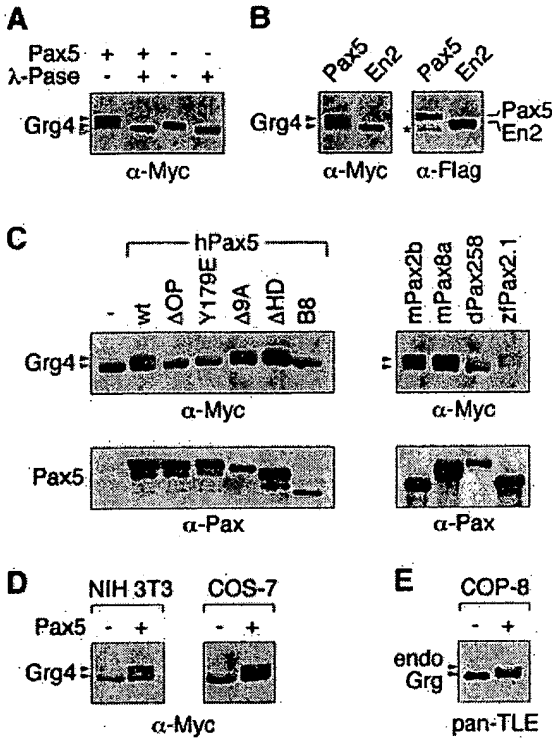


Fig. 6. Induced phosphorylation of Grg4 upon interaction with Pax5. (A) Pax5-dependent phosphorylation of Grg4. Pax5 (where indicated) and Myc-tagged Grg4 were transiently expressed in COP-8 cells for 48 h. Grg4 was then precipitated from whole-cell lysates with a monoclonal anti-Myc antibody, incubated with λ protein phosphatase (λ-Pase) and then analyzed by Western blotting with a polyclonal anti-Myc antibody. (B) Specificity of the Grg4 phosphorylation. Myc-tagged Grg4 and Flag-tagged Pax5 or En2 proteins were co-expressed in COP-8 cells followed by Western blot analysis with anti-Myc and anti-Flag antibodies, respectively. The asterisk denotes a cross-reacting protein. (C) The phosphorylation of Grg4 depends on direct interaction with Pax2/5/8 proteins. The Pax proteins indicated were co-expressed with Myc-tagged Grg4 in COP-8 cells followed by Western blotting with polyclonal anti-Myc and anti-paired domain antibodies. (D) Phosphorylation of Myc-tagged Grg4 in NIH 3T3 and COS-7 cells co-expressing Pax5. (E) Endogenous (endo) Grg proteins are phosphorylated in Pax5-expressing COP-8 cells, as shown by Western blot analysis with a pan-TLE (Grg) antibody (Stifani *et al.*, 1992).

expressed mutant Pax5 proteins with Myc-tagged Grg4 in transiently transfected COP-8 cells, followed by Western blot analysis. Indeed, deletion of the Pax5 transactivation domain (B8) as well as mutation of the octapeptide motif (ΔOP, Y179E) prevented further phosphorylation of the Grg4 protein (Fgure 6C). In contrast, mutation of other Pax5 domains (Δ9A or ΔHD) had no effect on Grg4 phosphorylation (Figure 6C), indicating that the integrity of the two Grg4 interaction domains in Pax5 is essential for this additional modification to occur. Moreover, the Pax5-induced phosphorylation of Grg4 was also observed in transfected NIH 3T3 fibroblasts as well as in COS-7 kidney cells, and thus represents a cell type-independent phenomenon (Figure 6D). Importantly, even endogenous Grg proteins underwent phosphorylation in COP-8 cells overexpressing Pax5 (Figure 6E). En2, another Groucho-interacting transcription factor (Jiménez *et al.*, 1997), was, however, unable to induce additional phosphorylation of

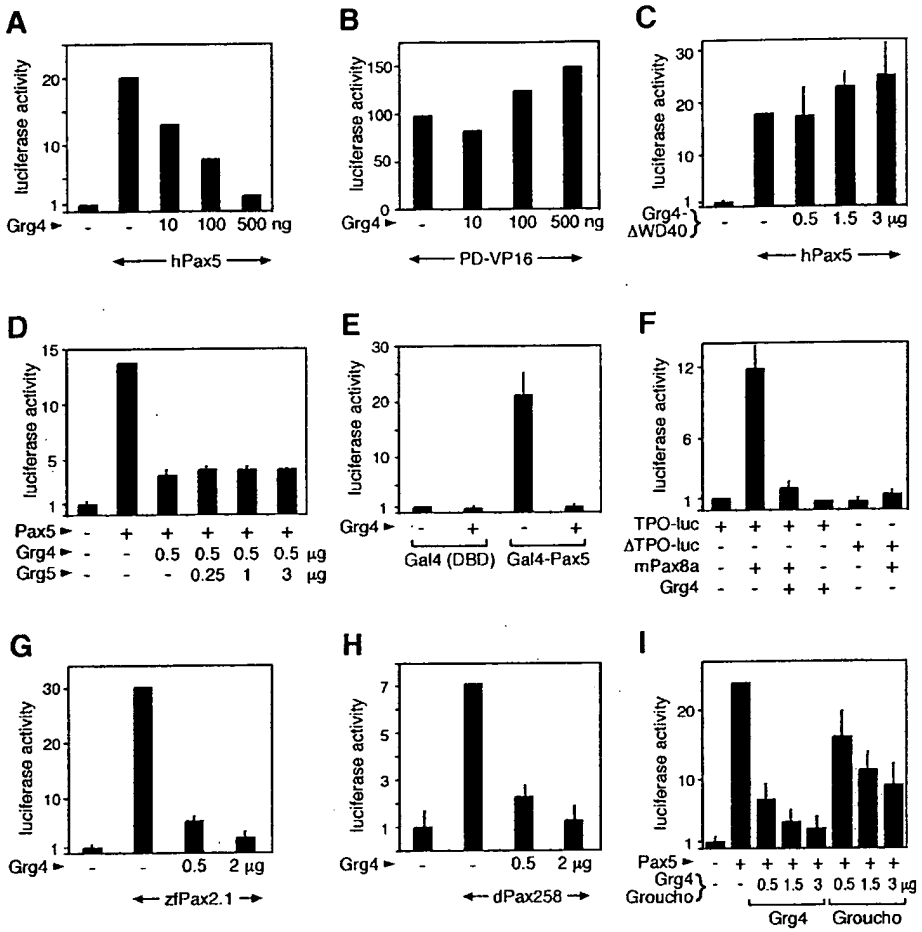


Fig. 7. Grg4 represses the transcriptional activity of Pax5. The Pax expression vector (0.5 μg), luciferase genes *luc-CD19* (5 μg) and pRL-SV40 (0.4 μg), and increasing amounts of the indicated Grg expression vector were used for transient transfection of SP2/0 cells (A–D and G–I). The amount of expression plasmid was equalized by the addition of pKW2T, and all data were evaluated as described in the legend to Figure 3A. Normalized luciferase values of one representative experiment (A and B) or three independent transfections (C–I) are shown relative to the luciferase activity measured in the absence of the Pax protein. (**A**) Repression of the Pax5 transactivation function by Grg4. (**B**) Failure of Grg4 to repress the function of a paired domain (PD)-VP16 protein. (**C**) Inability of the Grg4-ΔWD40 protein to repress the transcriptional activity of Pax5. (**D**) Grg5 fails to antagonize Grg4-mediated repression of Pax5 activity. (**E**) Repression of a chromatinized reporter gene by Grg4. Expression vectors (1 μg) encoding Gal4–Pax5 or the Gal4 DNA-binding domain (DBD) were transfected together with a Grg4 expression plasmid (0.5 μg) into U2-OS cells containing an integrated luciferase gene, followed by luciferase analysis as described (Alkema *et al.*, 1997). (**F**) Grg4-mediated repression of the rat *TPO* promoter. HeLa cells were transfected with mPax8a (0.1 μg) and Grg4 (0.5 μg) expression plasmids and the luciferase gene *TPO-luc* (2.5 μg) and pRL-SV40 (10 ng). Δ*TPO-luc* contains a mutated Pax8-binding site (Zannini *et al.*, 1992). (**G** and **H**) Grg4-mediated repression of the transcriptional activity of zebrafish zfPax2.1 and *Drosophila* dPax258. (**I**) Repression of the Pax5 transactivation function by *Drosophila* Groucho.

Grg4 (Figure 6B). Hence, this phosphorylation reaction cannot be promoted by all Groucho-binding proteins.

Grg4 represses the transcriptional activity of Pax5

As Groucho proteins are known to function as transcriptional corepressors (reviewed by Fisher and Caudy, 1998; Parkhurst, 1998), we investigated whether full-length Grg4 is able to modulate the transcriptional activity of Pax5. We thus studied the effect of Grg4 on the Pax5-dependent transcription of the reporter gene *CD19-luc* in transiently transfected plasmacytoma cells. As shown in Figure 7A, the Grg4 protein repressed Pax5-mediated activation of this reporter gene in a concentration-dependent manner. This repression was specific, as the transcriptional activity of a chimeric protein consisting of the VP16 transactivation domain fused to the Pax5 paired domain was not affected by Grg4 (Figure 7B). Interestingly, a Grg4 protein lacking all WD40 repeats failed to repress the activity of Pax5 (Figure 7C) despite the fact that this mutant Grg4 protein binds Pax5 with an efficiency similar to full-length Grg4 (Figure 4B). A fifth member of the *Grg* family, *Grg5*, codes for a short 197-amino-acid protein consisting only of the Q and SP domains (Mallo *et al.*, 1993). This small protein is able to antagonize Grg4-dependent repression by the transcription factors TCF and Blimp-1 (Roose *et al.*, 1998; Ren *et al.*, 1999). However, the Grg5 protein could not reverse Grg4-mediated repression of the Pax5 transactivation function (Figure 7D). In conclusion, Grg4 can repress the transcriptional activity of Pax5 efficiently by a mechanism that depends on the integrity of the WD40 repeats and is insensitive to the dominant-negative action of Grg5.

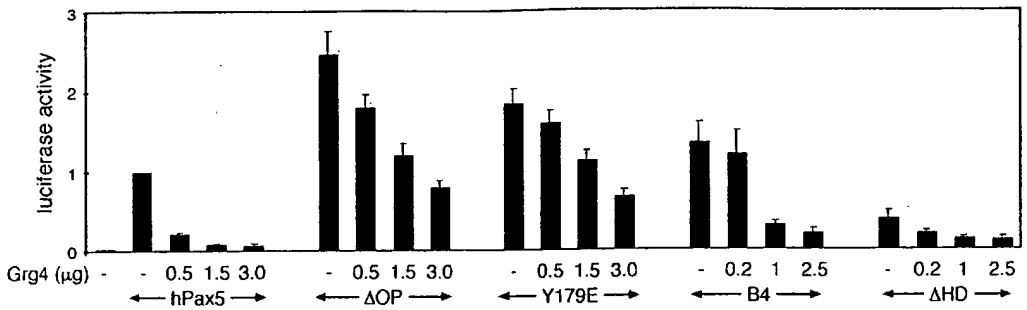


Fig. 8. The octapeptide motif of Pax5 mediates repression by Grg4. Transient transfection experiments with the expression plasmids indicated were performed in SP2/0 cells and subsequently evaluated as described in the legend to Figure 7.

We next examined whether Grg4 can also repress Pax5-mediated activation of a reporter gene that is stably inserted in the genome. For this experiment, we used a human osteosarcoma cell line that contains an integrated luciferase gene under the control of a thymidine kinase promoter and five upstream Gal4-binding sites (Alkema *et al.*, 1997). Luciferase expression was stimulated in these cells ~20-fold by a Gal4–Pax5 fusion protein relative to the Gal4 DNA-binding domain alone (Figure 7E). Co-expression of Grg4 efficiently inhibited transactivation by Gal4–Pax5 (Figure 7E), indicating that packaging of a reporter gene into chromatin still results in Grg4-mediated repression of Pax5 activity.

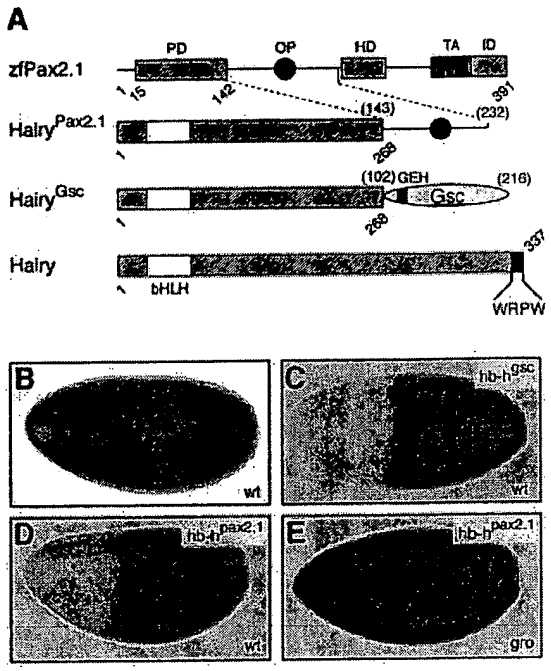
Although several B cell-specific target genes of Pax5 are known, they all contain TATA-less promoters that are almost inactive in transient transfection assays (reviewed by Busslinger and Nutt, 1998). To study Grg4-mediated repression of a naturally occurring Pax target gene, we analyzed the promoter of the rat thyroperoxidase (*TPO*) gene, which contains a Pax8-binding site immediately upstream of a TATA-box (Zannini *et al.*, 1992). A luciferase gene under the control of the *TPO* promoter was strongly activated by Pax8 in transiently transfected HeLa cells, whereas a promoter mutation inactivating the Pax8-binding site (Δ) prevented transcriptional stimulation (Figure 7F) (Zannini *et al.*, 1992). Co-expression of Grg4 efficiently repressed the Pax8-dependent activation of the *TPO* promoter, whereas Grg4 expression on its own did not affect basal promoter activity (Figure 7F). In summary, these data demonstrate that Pax5 and Pax8 can efficiently recruit Grg4 to artificial or naturally occurring promoters, which results in repression of gene transcription.

The octapeptide of Pax5 mediates repression by Grg4

To identify the domains of Pax5 that confer Grg4-mediated repression, we analyzed the transcriptional activity of several mutant Pax5 proteins in transiently transfected plasmacytoma cells. In the absence of exogenous Grg4 protein, the mutant proteins already differed in their transactivation potential (Figure 8). A Pax5 protein lacking the partial homeodomain (ΔHD) was 2- to 3-fold less active than the wild-type protein, whereas the transcriptional activity of the octapeptide deletion mutant (ΔOP) was increased consistently by a factor of 2–3 (Figure 8). Western blot analysis indicated, however, that these differences in transcriptional activity were not caused by different expression levels of the mutant Pax5 proteins (data not shown). The lower activity of the Pax5-ΔHD protein could be explained by our recent finding that the partial homeodomain is an interaction motif for the TATA-binding protein and may thus facilitate recruitment of the basal transcription factor complex TFIID to the promoter (Eberhard and Busslinger, 1999). Moreover, RNase protection analyses revealed that the known mouse *Grg(1/3a/4)* genes are constitutively expressed at all stages of B-cell development as well as in the plasmacytoma cells analyzed (data not shown). Hence, endogenous Grg proteins may interact with Pax5 and reduce its activity in transfected cells, whereas the increased transactivation potential of the Pax5-ΔOP protein could reflect the loss of Grg binding.

Consistent with this hypothesis, expression of exogenous Grg4 protein could repress the transcriptional activity of the Pax5-ΔOP protein only 2-fold, in contrast to the wild-type Pax5 protein, whose transactivation function was completely inhibited (Figure 8). Even at the highest Grg4 concentration, the Pax5-ΔOP protein was transcriptionally as active as the wild-type Pax5 protein in the absence of any exogenous Grg4 protein. Moreover, the single amino acid substitution Y179E prevented Grg4-mediated repression of Pax5 activity to the same extent as deletion of the entire octapeptide sequence (Figure 8), further demonstrating that the octapeptide motif of Pax5 is essential for *in vivo* binding and thus recruitment of the corepressor Grg4. The residual 2-fold repression of the octapeptide mutants by Grg4 suggests that the two proteins can still bind to each other weakly *in vivo* through the second, still intact interaction involving the transactivation domain of Pax5 and the Q domain of Grg4 (Figure 3B).

The C-terminal sequences of Pax5 contain an inhibitory domain that negatively regulates the adjacent transactivation region of Pax5 in most cell lines including the J558L cells, but not in SP2/0 cells (Dörfler and Busslinger, 1996). A mutant Pax5 protein (B4) lacking this inhibitory domain was still repressed efficiently by Grg4 in both SP2/0 and J558L cells (Figure 8; data not shown). Furthermore, Grg4 was also able to repress the low transcriptional activity of the homeodomain deletion mutant Pax5-ΔHD (Figure 8A). These data therefore indicate that neither the C-terminal inhibitory sequence nor the partial homeodomain of Pax5



F

construct	females	males
hb-h^{gsc}	0	59
hb-h$^{pax2.1}$	61	54

Fig. 9. The octapeptide motif functions as a repression domain in *Drosophila* embryos. (A) Schematic diagram of the Hairy derivatives used in the *Sxl* repression assay. The Hairy$^{Pax2.1}$ protein was generated by fusing the Hairy sequences at amino acid 268 to a 90-amino-acid sequence (143–232) of the zebrafish Pax2.1 protein encompassing the conserved octapeptide motif (Pfeffer *et al.*, 1998). The Hairy^{Gsc} protein containing the eh1/GEH motif of Goosecoid (Gsc) was described previously (Jiménez *et al.*, 1999). (B–E) *Sxl* repression by chimeric Hairy proteins in female blastoderm embryos of *Drosophila*. The Hairy^{Gsc} (C) and Hairy$^{Pax2.1}$ (D and E) proteins were expressed under the control of the *hunchback* (*hb*) promoter in wild-type (wt, C and D) or *gro*$^{E48mat-}$ mutant embryos (E). Sxl expression was analyzed in transgenic (C–E) and control (B) female embryos by whole-mount staining with a monoclonal antibody detecting the female-specific form of Sxl (Bopp *et al.*, 1991). (F) Effects of Hairy^{Gsc} and Hairy$^{Pax2.1}$ expression on female viability. Wild-type females were crossed with *hb* transgenic males, and the number of viable progeny is indicated.

are essential for Grg4-mediated repression of the Pax5 transactivation function.

Evolutionary conservation of the interaction between Groucho and Pax proteins

Three *groucho*-related genes coding for full-length Grg proteins (Grg1, 3a and 4) have been identified to date in the mouse genome (Koop *et al.*, 1996; Leon and Lobe, 1997). Using transient transfection assays, we have shown that all three murine Grg proteins are phosphorylated in a Pax5-dependent manner and can repress the transcriptional activity of Pax5 efficiently (data not shown). Even the distantly related Groucho protein of *Drosophila* was able to interact with Pax5 (Figures 3A and 5) and to down-modulate the activity of this transcription factor in heterologous mammalian cells (Figure 7I).

Furthermore, GST pull-down assays demonstrated that the mouse Pax8 and *Drosophila* Pax258 proteins can bind full-length Grg4 with an affinity similar to that of human Pax5 (Figure 4C). Moreover, the transcriptional activity of the mouse Pax8, zebrafish Pax2.1 and *Drosophila* Pax258 proteins could be repressed efficiently by Grg4 in transfected plasmacytoma cells (Figure 7F, G and H). These different Pax proteins were also able to promote additional phosphorylation of Grg4 in transfected COP-8 fibroblasts (Figure 6C). Collectively, these data demonstrate, therefore, that the interaction between distantly related members of the Pax2/5/8 and Groucho protein families has been conserved in evolution.

The octapeptide motif functions as a Groucho-dependent repression domain in Drosophila embryos

Inspired by the high evolutionary conservation of the Groucho–Pax2/5/8 protein interaction, we next investigated whether the octapeptide motif can function *in vivo* as a repression domain during *Drosophila* development. For this, we took advantage of a repression assay that is based on the transcriptional regulation of the *Sex lethal* (*Sxl*) gene in *Drosophila* embryos (Parkhurst *et al.*, 1990). Sxl is a key regulator of sex determination and dosage compensation, whose transcription is initiated only in female blastoderm embryos. In male embryos, *Sxl* expression is prevented by the transcriptional repressor Deadpan (Dpn), which is a member of the Hairy-related basic helix–loop–helix (bHLH) protein family. The negative effect of Dpn can be mimicked in female embryos by ectopic expression of the related Hairy protein at the time of sex determination (Parkhurst *et al.*, 1990). Premature Hairy expression under the control of the *hunchback* (*hb*) promoter represses *Sxl* transcription in the anterior part of female embryos, which leads to female-specific lethality (Parkhurst *et al.*, 1990). Repression of *Sxl* by Hairy depends on the interaction of its C-terminal WRPW motif with Groucho (Paroush *et al.*, 1994) and, consequently, does not occur in embryos deprived of maternal Groucho function (Jiménez *et al.*, 1997). Moreover, substitution of the C-terminal Hairy sequences by a heterologous repression domain still leads to down-regulation of *Sxl* expression, thus providing a convenient assay for studying Groucho-dependent repression domains *in vivo* (Jiménez *et al.*, 1999).

We used this assay to examine the *in vivo* function of the octapeptide motif by replacing the C-terminal region of Hairy with a sequence encompassing the 90 amino acids located between the paired domain and partial homeodomain of zfPax2.1 (Figure 9A). The octapeptide motif is the only conserved element that is shared between this zebrafish Pax2.1 sequence and the corresponding region of the *Drosophila* Pax258 protein (Czerny *et al.*, 1997). Expression of the chimeric Hairy$^{Pax2.1}$ protein under the control of the *hb* promoter resulted in significant reduction of Sxl expression in the anterior half of transgenic female embryos (Figure 9D) compared with the uniform Sxl staining of wild-type embryos (Figure 9B). Moreover, the repression of *Sxl* by Hairy$^{Pax2.1}$ was dependent on Groucho, as it was not observed in embryos lacking

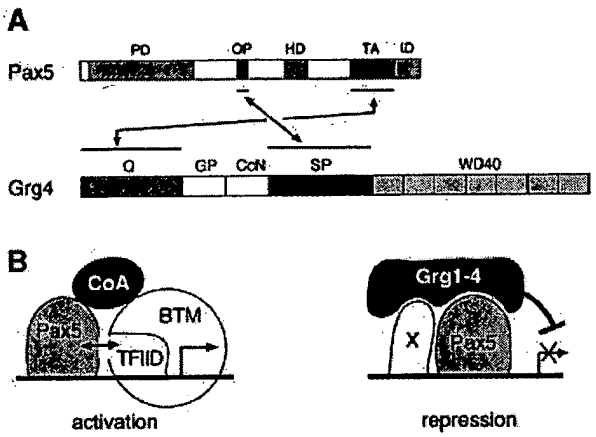


Fig. 10. Model of Grg4 recruitment by Pax5 (BSAP). (A) Summary of the identified interactions between Pax5 and Grg4. (B) Model of Grg-mediated gene repression by Pax5. As different members of the Grg family are co-expressed with Pax5 throughout midbrain and B-cell development (Koop *et al.*, 1996; D.Eberhard, unpublished data), we hypothesize that Pax5 can stably recruit Grg proteins to a specific promoter only in collaboration with a second Grg-binding transcription factor (X). CoA, coactivator protein(s); BTM, basal transcription machinery.

maternal *gro* function (Figure 9E; Materials and methods). However, the Hairy$^{Pax2.1}$ protein was clearly less active in repressing the *Sxl* gene than a Hairy^{Gsc} protein (Figure 9C) containing the GEH motif of Goosecoid (Gsc) as a potent repression domain (Jiménez *et al.*, 1999). This difference in repression activity is also reflected by the fact that ectopic expression of Hairy^{Gsc} caused female lethality, whereas Hairy$^{Pax2.1}$ did not significantly affect female viability (Figure 9F). These data indicate that the octapeptide motif of the zebrafish Pax2.1 protein can function as a weak Groucho-dependent repression domain in *Drosophila* embryos.

Discussion

Commitment to the B-lymphoid lineage critically depends on the repression of lineage-inappropriate genes by the transcription factor Pax5 (BSAP) (Nutt *et al.*, 1999). By identifying Grg4 as a corepressor of Pax5, we have now elucidated a molecular mechanism by which Pax5 can act as an active repressor of gene transcription. Grg4 was shown to interact with Pax5 *in vitro* in GST pull-down assays as well as *in vivo* in yeast and various mammalian cell types. Grg4 and Pax5 both contain two separate interaction domains that cooperate together in protein binding (Figure 10A). As a consequence, Grg4 could repress the transcriptional activity of Pax5 efficiently in transiently transfected cells. Similar protein interactions resulting in repression were also observed between distantly related members of both the Pax2/5/8 and Groucho protein families. Moreover, Grg4 also interacts with Pax1, Pax3 and Pax6 (D.Eberhard, unpublished data), which are representative members of the other three Pax subfamilies. Hence, Groucho proteins appear to act as corepressors of all Pax transcription factors. It has recently been suggested that Pax3 mediates gene repression by recruiting the corepressors HIRA or Daxx, respectively (Magnaghi *et al.*, 1998; Hollenbach *et al.*, 1999). In addition, we have shown that the Rb protein can interact with the partial homeodomain of Pax5 (Eberhard and Busslinger, 1999). However, none of these proteins (HIRA, Daxx or Rb) was able to repress the transcriptional activity of Pax5 (data not shown), suggesting that the Pax transcription factors exert their repression function primarily by recruiting corepressors of the Groucho family.

Two distinct interactions contribute to the formation of the Grg4–Pax5 complex

Several transcription factors are known to interact with Groucho proteins and, wherever studied, a single domain in each protein was responsible for this interaction (reviewed by Fisher and Caudy, 1998; Parkhurst, 1998). Pax5 is thus the first example of a transcription factor that relies on two separate interactions for Groucho binding (Figure 10A). One of these interactions is mediated by the N-terminal Q domain of Grg4 and the C-terminal transactivation domain of Pax5. Detailed *in vitro* mutagenesis indicated that the integrity of the entire Q domain is essential for Pax5 binding (data not shown). The highly conserved Q domain contains two leucine zipper-like motifs that are both required for tetramerization of Groucho proteins (Chen *et al.*, 1998). In addition, the Q domain is sufficient to mediate binding of the Blimp-1 (PRDI-BF1) (Ren *et al.*, 1999) and TCF proteins (Roose *et al.*, 1998). Consequently, the short Grg5 protein consisting only of the Q and SP domains (Mallo *et al.*, 1993) can act as a dominant-negative protein to reverse Grg4-mediated repression by these two transcription factors (Roose *et al.*, 1998; Ren *et al.*, 1999). In contrast, the interaction between the Q domain of Grg4 and the transactivation domain of Pax5 is too weak to be detected in *in vitro* binding assays. In addition, the Grg5 protein is unable to antagonize Grg4-mediated repression of the Pax5 transcriptional activity. Both findings are consistent with the notion that two separate but cooperative interactions are required for efficient formation of the Grg4–Pax5 complex.

The second interaction that contributes to complex stability involves the SP domain of Grg4 and the octapeptide motif of Pax5 (Figure 10). The SP domain, which was previously implicated in the interaction between Groucho and Hairy-related bHLH proteins (Paroush *et al.*, 1994; Jiménez *et al.*, 1997), was again insufficient to mediate Pax5 binding, and even three copies of the octapeptide motif linked to a Gal4 protein failed to interact with Grg4 in *in vitro* binding assays (data not shown). The octapeptide sequence was identified 10 years ago as a conserved motif of most Pax proteins (Burri *et al.*, 1989). Here, we show that this short amino acid sequence constitutes a protein interaction domain, is required in mammalian cells for Grg4-mediated repression of the Pax5 transactivation function and acts as a weak Groucho-dependent repression domain in *Drosophila* embryos. The octapeptide motif is related in sequence to the eh1/GEH region that is found in several homeodomain transcription factors (Noll, 1993; Smith and Jaynes, 1996). Consistent with this sequence similarity, the Y179E substitution in the octapeptide motif abrogated Groucho protein binding (Figure 4) in analogy to the equivalent F-to-E substitution in the eh1/GEH region (Jiménez *et al.*, 1999). Apart from

these similarities, the octapeptide differs in several aspects from the eh1/GEH region. First, the eh1/GEH sequence is both necessary and sufficient for Groucho binding and transcriptional repression (Jiménez *et al.*, 1999), in contrast to the octapeptide motif (this study). Secondly, the eh1/GEH region functions as a more potent Groucho-dependent repression domain in *Drosophila* embryos than the octapeptide sequence (Jiménez *et al.*, 1997, 1999). Thirdly, the WD40 repeats of Groucho are essential for binding of the eh1/GEH region (Jiménez *et al.*, 1997), whereas the SP domain of Grg4 seems to mediate interaction with the Pax5 octapeptide motif. However, the WD40 repeats of Grg4 are still required for repression of the Pax5 activity, suggesting that these conserved protein interaction motifs recruit additional factors into the Grg4–Pax5 complex.

Pax5-dependent phosphorylation of Grg proteins

Groucho proteins are known to be constitutively phosphorylated on serine and threonine residues (Husain *et al.*, 1996). Hence, we were surprised to see that the Grg proteins undergo further phosphorylation upon Pax5 binding. This phosphorylation reaction is strictly dependent on the interaction with Pax5 and appears to be catalytic, as the majority of the Grg proteins become phosphorylated in cells overexpressing Pax5 (Figure 6) despite the fact that only a small proportion of these proteins could be co-precipitated with Pax5, possibly due to the relatively low stability of the Grg4–Pax5 complex (Figure 5). In contrast, the En2 protein, which interacts efficiently with Grg4 *in vivo* (data not shown), was unable to induce this additional phosphorylation, thus revealing a remarkable selectivity with regard to the Groucho-binding transcription factors involved in this phenomenon. Two different hypotheses could account for these observations. Pax5, in contrast to En2, binds Grg4 via two different interactions that may induce a conformational change in Grg4 and thus render cryptic phosphorylation sites accessible to a constitutively active kinase. Alternatively, Pax5 may specifically recruit a kinase into the Groucho complex in analogy to the homeodomain protein NK-3, which interacts simultaneously with Groucho and the nuclear kinase HIPK2 (Choi *et al.*, 1999). Interestingly, signaling of the Torso receptor via the MAP kinase pathway has been implicated in antagonizing Groucho-mediated gene repression at the terminal pole regions of the *Drosophila* embryo (Paroush *et al.*, 1997). Torso signaling does not, however, interfere with all Groucho functions, as it specifically affects only certain Groucho-containing complexes (Paroush *et al.*, 1997), in analogy to the transcription factor selectivity observed for the induced Grg phosphorylation described in this study.

Grg-dependent transcriptional repression by Pax5

The different Groucho-binding transcription factors can be grouped into three classes according to their mode of Grg protein recruitment. The first class consists of active repressors comprising the Hairy-related bHLH proteins (Paroush *et al.*, 1994), the homeodomain proteins En, Gsc and NK-3 (Jiménez *et al.*, 1997, 1999; Choi *et al.*, 1999) and the zinc finger proteins Hkb and Blimp-1 (Goldstein *et al.*, 1999; Ren *et al.*, 1999). All of these negative regulators utilize short sequence motifs to recruit Groucho proteins stably and thus act as constitutive repressors of transcription. In contrast, the HMG-box proteins of the TCF family bind Grg proteins in a manner that is regulated by Wnt signaling. These TCF proteins interact with Groucho proteins and thus function as transcriptional repressors in the absence of a Wnt signal. Upon signal transduction, the TCF proteins are converted into transcriptional activators by displacement of the Grg protein with the coactivator β-catenin (Roose *et al.*, 1998). The third class consists of intrinsic transcriptional activators, as exemplified by the Dorsal and Runt proteins (Aronson *et al.*, 1997; Dubnicoff *et al.*, 1997). These transcription factors on their own are unable to recruit Groucho proteins to their target genes *in vivo*. Instead, they require the assistance of other Groucho-interacting transcription factors to recruit Grg proteins, and thus function as repressors only in a regulatory context-dependent manner (Valentine *et al.*, 1998).

Pax5 is known simultaneously to activate B cell-specific genes (such as *CD19*) and to repress lineage-inappropriate genes (such as *M-CSF-R*) in the same early B-lymphoid progenitor cell (Nutt *et al.*, 1997, 1998, 1999). Hence, Pax5 appears to belong to the class of transcription factors that are converted from activators to repressors by co-recruitment of Groucho proteins in a context-dependent manner (Figure 10B). The transcriptional activity of Pax5 was, however, repressed by Grg proteins in transient transfection assays, even though the artificial promoter of the reporter gene contained only Pax-binding sites. This apparent contradiction could be explained by the fact that the high expression levels attained in transiently transfected cells force the Grg4 protein to interact with Pax5. In addition, the Pax5 proteins bound to the multimerized sites in the reporter gene may cooperate in Grg4 binding, thus mimicking a co-recruitment paradigm. Pax5 is known to negatively regulate the activity of the 3′α enhancer present in the immunoglobulin heavy chain locus (reviewed by Busslinger and Nutt, 1998). Interestingly, the Pax5-dependent down-regulation of this enhancer not only depends on the presence of a Pax5 recognition sequence, but also requires the integrity of adjacent transcription factor-binding sites (Singh and Birshtein, 1996). By reconstituting the 3′α enhancer activity in heterologous cells, we have recently been able to demonstrate that Pax5 requires cooperation with other transcription factors to repress this enhancer in a Grg4-dependent manner (Y.Linderson, D.Eberhard, S.Pettersson and M.Busslinger, unpublished data). Hence, context-dependent recruitment of Groucho proteins may be a general mechanism that converts Pax proteins from activators to repressors of gene transcription.

Materials and methods

DNA constructs

All cDNAs were cloned into the eukaryotic expression vector pKW2T (Dörfler and Busslinger, 1996). The mouse *Grg4* cDNA was amplified by RT–PCR from RNA of 9.5/10.5-day-old embryos with the primers 5′-CCCAAGCTTACCATGGTTCCGCAGACGCGC-3′ and 5′-GCTCTAGATGCTATGAGGAGGAGTCCAG-3′. Likewise, the mouse *Grg5* cDNA was amplified with the primers 5′-CCCAAGCTTACCATGATGTTTCCGCAAAGC-3′ and 5′-CAGCCAGAACCAGGACTG-3′. The *Drosophila groucho* cDNA was isolated by RT–PCR from embryonic RNA using the primers 5′-ACATGACCATGGTTCCCT-

CACCGGTGCGCC-3′ and 5′-ACCCAAGCTTGGATCCTTTTGTTT-TACTGCCGATGCT-3′. The sequence context surrounding the start codon of the *Drosophila Pax258* cDNA (Czerny *et al.*, 1997) was optimized by inserting the oligonucleotide 5′-GCGGAATTCCA-CCATGGGCAGTATTTCGGGTGATGGTCATGGAGGCGTTAATC-AAC-3′ by PCR. Myc or Flag epitope tags were added by PCR at the N-terminus of the expression constructs. The Pax5 mutants ΔOP, Δ9A and Y179E were generated by PCR-based mutagenesis in addition to the previously described mutants B4, B8, B9 and ΔHD (Dörfler and Busslinger, 1996; Eberhard and Busslinger, 1999). A PCR fragment encoding amino acids 146–391 of Pax5 was inserted into the expression vector pPuroGal4 (Alkema *et al.*, 1997) to obtain plasmid pPuroGal4-Pax5, which was used for transfection of U2-OS cells. VP16 and VP16-Grg4 expression plasmids were generated by inserting the *Hin*dIII–*Eco*RI fragment of the yeast vector pVP16 or the respective two-hybrid clone into pKW2T. The VP16-Gro construct contained a PCR fragment coding for amino acids 1–158 of *Drosophila* Groucho in the *Not*I site of pVP16. All GST–Grg4 constructs were obtained by insertion of the respective PCR fragments into pGEX vectors (Amersham Pharmacia Biotech).

Cell lines

The murine plasmacytoma cells SP2/0 and J558L, murine COP-8 and NIH 3T3 fibroblasts, monkey COS-7 kidney cells, and human U2-OS osteosarcoma and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. These cell lines do not express endogenous Pax5 (Dörfler and Busslinger, 1996).

Yeast two-hybrid screen

The Matchmaker™ two-hybrid system (Clontech) was used to screen for interaction partners of Pax5. Briefly, a PCR fragment of human Pax5 (amino acids 146–391) was inserted into the *Bam*HI site of the Gal4 DNA-binding domain plasmid pGBT9. This construct was transformed into the yeast strain HF7c (Clontech). Single colonies grown on synthetic medium lacking tryptophan were transformed with a mouse cDNA-VP16 fusion library prepared from RNA of 9.5/10.5-day-old embryos (Hollenberg *et al.*, 1995). One colony out of 3.2×10^6 transformants activated the *HIS3* gene in a Pax5 bait-dependent manner, as it contained a VP16-Grg4 cDNA fragment.

GST pull-down assay

Purified GST fusion proteins (2–5 μg) immobilized on glutathione–Sepharose beads were incubated for 2 h at 4°C with *in vitro* synthesized ^{35}S-labeled protein (5 μl) in buffer BC100 (200 μl) supplemented with 2 mg/ml bovine serum albumin (BSA), 0.2% NP-40 and 100 μg/ml ethidium bromide, then washed extensively, eluted and analyzed by SDS–PAGE as previously described (Eberhard and Busslinger, 1999).

Cell transfection assay

J558L and SP2/0 cells were transiently transfected by electroporation with the firefly luciferase reporter gene *luc-CD19*, the *Renilla* luciferase plasmid pRL-SV40 (Promega) and Pax5 and Grg4 expression plasmids, as described (Dörfler and Busslinger, 1996). The COP-8 and U2-OS cells were transfected with LipofectAMINE PLUS™ Reagent (Gibco-BRL) and HeLa cells with the FuGENE™ 6 Transfection Reagent (Boehringer Mannheim) according to the supplier's instructions. Luciferase activities were measured by the Dual-Luciferase™ reporter assay (Promega) in a Lumat LB 9507 bioluminescence counter (EG&G Berthold, Bad Wildbad, Germany).

Co-immunoprecipitation analysis

Transiently transfected COP-8 cells were lysed in buffer A [20 mM Tris–HCl pH 7.9, 120 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.2% NP-40, 1 mM dithiothreitol (DTT), 10% glycerol supplemented with 2 mM benzamidine hydrochloride, 0.1 mg/ml Pefabloc, 5 μg/ml each of pepstatin, leupeptin and aprotinin, 2 μg/ml each of antipain and chymostatin]. Lysates were incubated for 30 min on ice, cleared from cellular debris by centrifugation and subsequently mixed with 10 μl of anti-Flag M2 affinity beads (Sigma) for 2 h at 4°C under constant rotation. After extensive washing of the beads, the precipitated proteins were analyzed by SDS–PAGE and Western blotting using polyclonal anti-Myc and anti-Pax5 antibodies.

Generation of transgenic flies and analysis of Sxl expression

The *hb-h* $^{pax2.1}$ transgene was constructed and injected into *y w* embryos as described (Jiménez *et al.*, 1997). The analysis of several transformant lines yielded equivalent results. For the experiments shown in Figure 9, males carrying an insertion on the X chromosome were crossed with wild-type or mosaic *gro* females (see below), so that all female embryos inherited the *hb-h* $^{pax2.1}$ transgene. Embryos deprived of maternal *gro* function were obtained using the *gro*^{E48} allele and the *ovo*^{D}*-FLP-FRT* system as described (Jiménez *et al.*, 1999). This system generates homozygous mutant clones in the germline of heterozygous females, thus circumventing the lethality of homozygous *gro* females. Sxl staining was performed with a monoclonal antibody specific for the active form of the protein (Bopp *et al.*, 1991).

Accession number

The mouse *Grg4* cDNA has been submitted to DBBJ/EMBL/GenBank (accession No. AF229633).

Acknowledgements

We thank S.Stifani for anti-TLE antibodies, S.M.Hollenberg for the cDNA library, H.Clevers and R.Di Lauro for plasmids, M.van Lohuizen for U2-OS cells, and D.Ish-Horowicz for his support. D.E. was a recipient of a Marie Curie fellowship. This work was supported by the I.M.P. Vienna and, in part, by the Austrian Industrial Research Promotion Fund.

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Received December 21, 1999; revised and accepted March 16, 2000

Note added in proof

Sequence analysis of the mouse Grg4 protein with hidden Markov models derived from two different alignments of WD40 repeats [present in PFAM (Bateman *et al.*, 2000) and REP (Andrade *et al.*, 2000)] revealed five common hits with both alignments. The two models additionally located a sixth repeat at non-overlapping positions within Grg4. Hence, Grg proteins also contain seven WD40 repeats like other proteins containing WD40 motifs (Frank Eisenhaber, unpublished data).

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Dev Genes Evol (2000) 210:47–50

SHORT COMMUNICATION

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Hans-Henning Arnold

NKX2 gene expression in neuroectoderm but not in mesendodermally derived structures depends on sonic hedgehog in mouse embryos

Received: 27 May 1999 / Accepted: 14 July 1999

Abstract NKX2 genes in vertebrates encode a subfamily of homeodomain-containing transcription factors which regulate morphogenetic events and cell differentiation during embryogenesis. In mouse embryos several NKX2 genes are expressed in the ventral midline domains of the neuroectoderm, while other NKX2 genes are primarily expressed in the mesendoderm and mesendodermally derived organs, such as heart and gut. Within several patterning centers for tissue organization sonic hedgehog (Shh) is an important signal in the formation of ventral midline structures in vertebrate embryos. Here, we investigated the role of Shh in the embryonic expression of six different but closely related NKX2 genes in Shh null mutant mice. We found that expression of NKX2.1, NKX2.2, and NKX2.9 in neural domains requires Shh signaling, whereas NKX2.3, NKX2.5 and NKX2.6 expression in endoderm and mesoderm is independent of Shh.

Key words NKX2 gene expression · Sonic hedgehog mutant · Mouse development

Introduction

NKX genes of vertebrates are divided into subgroups according to their structural homology to four Drosophila NK genes. Six NK2/3-related genes have been identified in mouse and shown to be active during embryonic development and in the adult (for review see Harvey 1996). Some of the murine NKX2 genes are expressed primarily in the developing brain and neural tube, while others are transcribed in branchial arches and in mesendoderm giving rise to heart and gut. As many of the NKX2 expression domains in mouse embryos appear to be confined to the ventral midline regions, we wondered whether the ventralizing signal Shh may be involved in activating these genes. To determine the possible role of Shh in NKX2 gene expression in mouse, we examined homozygous Shh null mouse embryos (Chiang et al. 1996) by whole mount in situ hybridization with various NKX2 probes and compared the patterns with wild-type embryos of corresponding developmental stages.

Edited by R. Balling

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Materials and methods

E9.5 wild-type and homozygous Shh mutant mouse embryos were fixed in 4% paraformaldehyde/phosphate-buffered saline at 4°C overnight and subjected to whole mount in situ hybridization as described previously (Bober et al. 1994). Riboprobes for Shh, NKX2.2, NKX2.9, and NKX2.3 were generated as described by Pabst et al. (1997, 1998). NKX2.5 probe was made from the entire cDNA coding region. The NKX2.6 template was produced from cDNA of branchial arches from an E9.5 mouse embryo by reverse transcription-polymerase chain reaction with the following primers: upstream primer, ATGCTGTCGAGTCCTGTGGC; downstream primer, GGCTCGCATAGCTAGCGTCG. The cDNA template for the NKX2.1 probe (provided by R. Di Lauro) encoded nucleotides 1763–2291 of the rat TTF-1 cDNA (Guazzi et al. 1990).

Results and discussion

We and others have previously shown that many of the NKX2 genes in vertebrate embryos are expressed in patterns that either overlap or abut domains of Shh expression. These observations raise the possibility that NKX2 genes are generally involved in cell-type specification in response to Shh signaling. To test this hypothesis we performed in situ hybridizations on homozygous Shh mouse mutants with various NKX2 probes. Representative re-

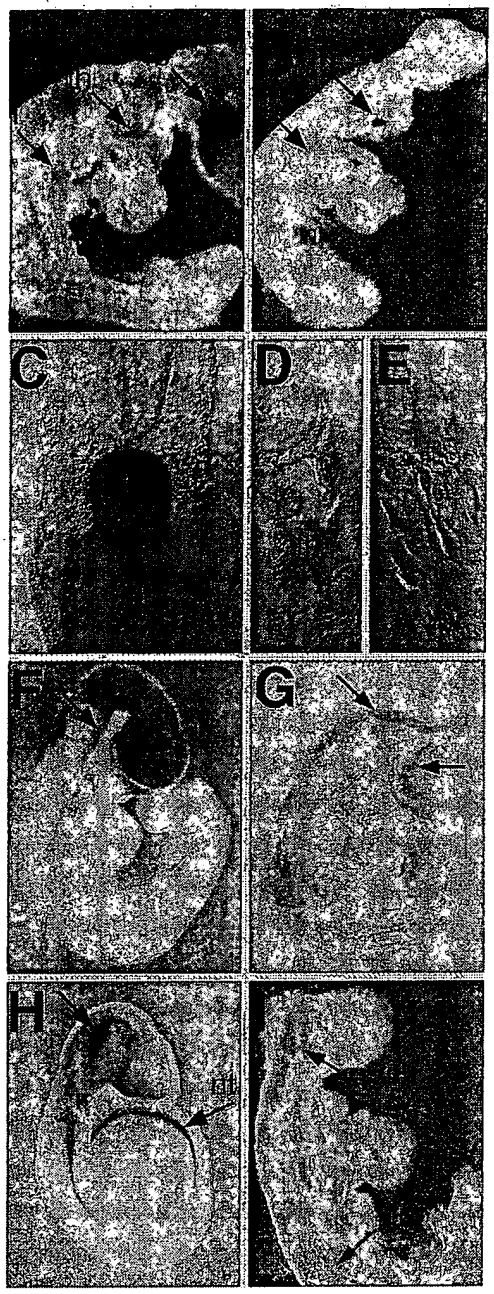

Fig. 1 Whole mount in situ hybridization and sections of E9.5 wild-type (**A,C,F,H**) and Shh mutant embryos (**B,D, E, G, I**) with NKX2.1 probe (**A–E**), NKX2.2 probe (**F,G**), and NKX2.9 probe (**H,I**). Expression domains in brain, thyroid and lung anlage, and neural tube are marked by *black arrows*. Expression missing in Shh mutant embryos is highlighted by *red arrows*. Transversal sections of the embryo shown in **A** and **B** are depicted in **C**, and **D** and **E**, respectively. NKX2.1 expression is shown in ventral forebrain of a wild-type mouse (**C**), and in the thyroid (**D**), and lung anlage (**E**) of mutant mice. The weak expression of NKX2.1 in lung and thyroid of the mutant mouse is unaltered compared to the wild type (data not shown) (*fb* forebrain, *th* thyroid gland, *l* lung, *nt* neural tube)

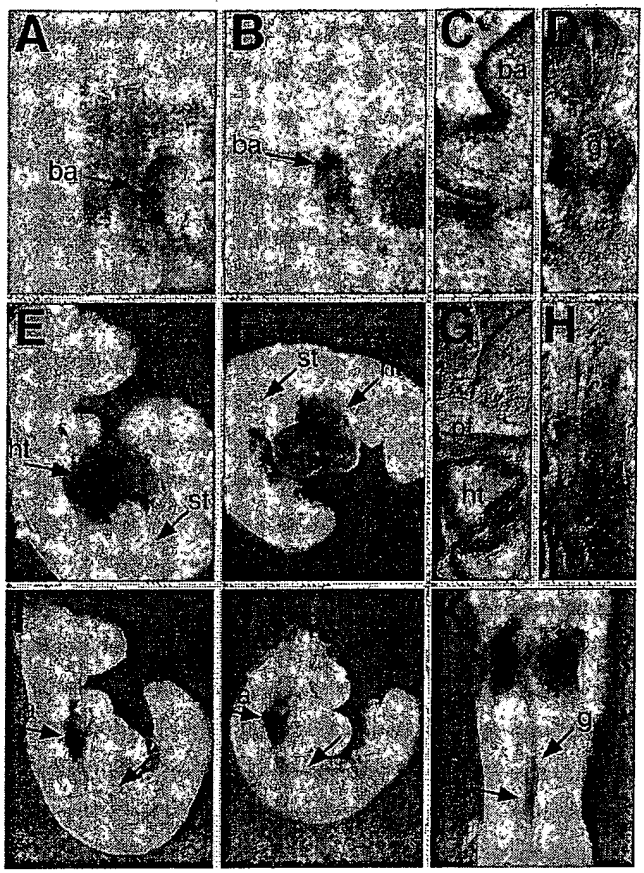

Fig. 2 Whole mount in situ hybridizations and sections of E9.5 wild-type (**A,E,I**) and Shh mutant embryos (**B–D,F–H,J,K**) with NKX2.3 (**A–D**), NKX2.5 (**E–H**) and NKX2.6 probes (**I–K**). Note that all expression domains (marked by *arrows*) in Shh mutants are unaltered as compared to wild type. Sections illustrate NKX2.3 expression in the branchial arch epithelium (**C**) and gut mesoderm (**D**), and NKX2.5 transcripts in myocardium, pharyngeal endoderm (**G**) and in the pylorus (**H**). NKX2.6 expression is detected in branchial arches and gut endoderm (**I–K**). The NKX2.6 transcript level appears to be slightly higher in a larger domain on the *left* side of the gut (**K**) (*ba* branchial arches, *g* gut, *ht* heart, *st* stomach, *pf* pharyngeal floor)

sults of embryonic NKX2 gene activities are illustrated for E9.5 mouse embryos (Figs. 1 and 2). In wild-type embryos transcripts for NKX2.1 accumulate in ventral forebrain (Fig. 1A, C), and in thyroid and lung anlagen (Fig. 1A). NKX2.2 mRNA is present also in forebrain and at low level in ventral hindbrain and neural tube (Fig. 1F). NKX2.9 is expressed in the brain and in ventral domains along the entire neural tube (Fig. 1H). These patterns appear in perfect agreement with previously published data (Guazzi et al. 1990; Price et al. 1992; Pabst et al. 1998). In contrast to wild-type embryos, E9.5 Shh null mutant embryos lack expression domains for all three genes in the neuroectoderm (red arrows in Fig. 1B, G, I), while NKX2.1 expression in the thyroid gland and lung is not affected by the Shh mutation (black arrows in Fig. 1B, D, E). The same results were obtained for other developmental stages (data not

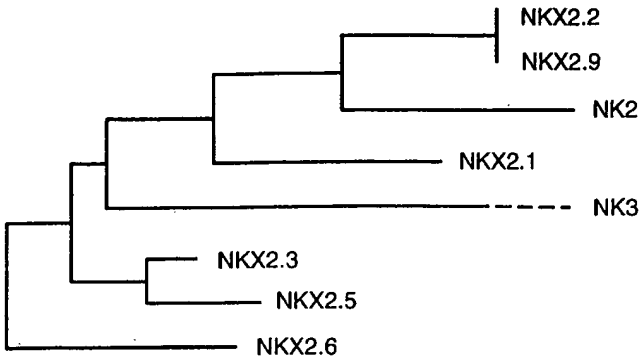

Fig. 3 Dendrogram of mouse NKX2 genes and their Drosophila homologs. The NKX genes, which are expressed in neuroectoderm and show dependence on Shh, form one subgroup (NKX2.1, NKX2.2, and NKX2.9), while the Shh-independent NKX genes form another subgroup (NKX2.3, NKX2.5 and NKX2.6). The relationship to Drosophila NK2 and NK3 genes is also shown. Sequences for this dendrogram were taken from Genbank database under the following accession numbers: NKX2.1, no. U19755; NKX2.2, no. U31566; NKX2.9, no. Y15741; NKX2.3, no. Y11117; NKX2.5, no. X75415; NKX 2.6, no. AF 045150; NK2, no. X87141; and NK3, no. L17133

shown), suggesting that Shh is absolutely required to activate these genes during embryonic development. Previous work has demonstrated that early telencephalic tissue responds to Shh signaling by expressing NKX2.1 (Ericson et al. 1995). Moreover, Pera and Kessel (1997) have recently shown that Shh expressed in prechordal plate of chick embryos is one of the signals involved in NKX2.1 gene activation in the ventral forebrain. NKX2.2 expression in embryonic forebrain of Zebrafish also appears responsive to Shh signals (Barth and Wilson 1995). A recent paper demonstrates that NKX2.2 has an essential role in interpreting graded Shh signals for the specification of neuronal identity in the ventral neural tube (Briscoe et al. 1999). Our results in the Shh mouse mutant confirm that all neuroectodermal domains of depend NKX2.1 and NKX2.2 gene expression on Shh and we extend these findings to the additional neural marker gene NKX2.9.

Unlike NKX2.1, NKX2.2, and NKX2.9, mouse NKX2.3, NKX2.5, and NKX2.6 are not expressed in neural structures, but are expressed in mesendodermal progenitor cells of heart and gut as well as in both mature organs throughout mouse development (Pabst et al. 1997; Lints et al. 1993; Biben et al. 1998). Regionalization of the gut involves epithelial-mesenchymal signaling, and Shh has been implicated as one of the signals (Roberts at al. 1998). Moreover, Shh mouse mutants display defects in foregut development (Litingtung et al. 1998), suggesting that Shh may affect at least some of the mesodermally expressed NKX2 genes. In E9.5 wild-type mouse embryos NKX2.3 is expressed in the epithelium of branchial arches and in mid- and hindgut mesoderm (Fig. 2A, and Pabst et al. 1997). The same spatial NKX2.3 expression is seen in $Shh^{-/-}$ mutant embryos of corresponding stages (Fig. 2B–D). NKX2.5 is highly transcribed in the pharyngeal floor, in the developing heart, and in the prospective pylorus (Fig. 2E). No alterations of NKX2.5 expression are observed in the Shh mutants (Fig. 2F–H). Likewise, NKX2.6 is expressed in branchial arch epithelium and gut endoderm at the level of the liver anlagen in both, wild-type and Shh mutant embryos (Fig. 2I–K). Significantly, NKX2.6 transcripts appear to accumulate asymmetrically in the pharyngeal endoderm with stronger expression on the left side, which is also maintained in the Shh mutant (Fig. 2K). Thus, no differences in the expression levels or regions of these NKX2 genes are seen between wild-type and Shh null mutant mice, indicating that Shh is not required for their activation. By inference, it can be concluded that these NKX genes, although expressed in domains with distinct boundaries along the antero-posterior gut axis, do not execute Shh-mediated patterning in the gut. They could, however, be part of a pre-patterning mechanism that leads to a regionalized response to the inductive influence of Shh in fore-, mid-, and hindgut (Roberts et al. 1998). In summary, one can distinguish two subgroups of NKX2 genes in mouse which are differentially dependent on Shh signaling. All expression domains of NKX2 genes in neuroectoderm require Shh, while all other sites of NKX2 gene activity are Shh independent.

Interestingly, the subdivision of NKX2 genes based on their expression is also reflected by their structural homology. Dendrograms compiled by sequence comparisons of the NKX2 homeodomains or the entire coding regions reveal that the Shh-dependent genes are more closely related to each other than to the members of the subgroup of Shh-independent NKX2 genes (Fig. 3). This observation may indicate that not only the structural properties of mouse NKX2 genes have been conserved after gene amplification but also at least some aspects of their expression control in the ventral domains of forebrain and neural tube.

Acknowledgements We would like to thank Drs. Hui Sheng Zheng and Heiner Westphal for Shh mutant embryos and Dr. R. Di Lauro for the TTF-1 cDNA. This work was supported by the Deutsche Forschungsgemeinschaft, SFB 271, TP A1 and Fonds der Chemischen Industrie.

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Different Levels of Repressor Activity Assign Redundant and Specific Roles to *Nkx6* Genes in Motor Neuron and Interneuron Specification

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Summary

Specification of neuronal fate in the vertebrate central nervous system depends on the profile of transcription factor expression by neural progenitor cells, but the precise roles of such factors in neurogenesis remain poorly characterized. Two closely related transcriptional repressors, Nkx6.2 and Nkx6.1, are expressed by progenitors in overlapping domains of the ventral spinal cord. We provide genetic evidence that differences in the level of repressor activity of these homeodomain proteins underlies the diversification of interneuron subtypes, and provides a fail-safe mechanism during motor neuron generation. A reduction in Nkx6 activity further permits V0 neurons to be generated from progenitors that lack homeodomain proteins normally required for their generation, providing direct evidence for a model in which progenitor homeodomain proteins direct specific cell fates by actively suppressing the expression of transcription factors that direct alternative fates.

Introduction

During the development of the vertebrate central nervous system, the assignment of regional identity to neural progenitor cells has a critical role in directing the subtype identity of post-mitotic neurons. Within the ventral half of the neural tube, the specification of progenitor cell identity is initiated by the long-range signaling activity of the secreted factor, Sonic hedgehog (Shh) (Briscoe et al., 2001; Briscoe and Ericson, 2001). Shh signaling appears to establish ventral progenitor cell identities by regulating the spatial pattern of expression of homeodomain transcription factors of the Nkx, Pax, Dbx, and Irx families (Ericson et al., 1997; Pierani et al., 1999; Briscoe et al., 2000). Members of all four gene families have been duplicated during evolution (Shoji et al., 1996; Wang et al., 2000; Hoshiyama et al., 1998; Peters et al., 2001), and the resulting homeodomain protein pairs are typically expressed in overlapping or nested domains within the neural tube (Briscoe and Ericson, 2001). Some of these homeodomain protein pairs have been proposed to have distinct, and others redundant, roles in spinal cord patterning (Mansouri and Gruss, 1998; Briscoe et al., 1999; Pierani et al., 2001), but the impact of such homeobox gene duplication on neuronal diversification has not been explored directly.

One unifying feature of this diverse array of progenitor homeodomain proteins is their subdivision into two general groups, termed class I and II proteins, on the basis of their mode of regulation by Shh signaling (Briscoe and Ericson, 2001). The class I proteins are constitutively expressed by neural progenitor cells, and their expression is repressed by Shh signaling, whereas neural expression of the class II proteins requires exposure to Shh (Ericson et al., 1997; Qiu et al., 1998; Briscoe et al., 1999, 2000; Pabst et al., 2000). Although the spatial pattern of expression of the class I proteins has revealed the existence of five ventral progenitor domains, class II proteins have been identified for only two of these domains (Briscoe et al., 2000), raising questions about the existence and identity of additional class II proteins. There is, however, emerging evidence that the combination of class I and II proteins that is expressed by neural progenitor cells directs the fate of their neuronal progeny. In support of this, misexpression of individual progenitor homeodomain proteins in the chick neural tube promotes the ectopic generation of neuronal subtypes, with a specificity predicted by the normal profile of progenitor homeodomain protein expression (Briscoe et al., 2000; Pierani et al., 2001). Conversely, the analysis of mouse mutants has provided genetic evidence that the activities of specific class I and II proteins are required to establish progenitor cell domains and to direct ventral neuronal fates (Ericson et al., 1997; Briscoe et al., 1999; Sander et al., 2000; Pierani et al., 2001).

The participation of progenitor homeodomain proteins in the conversion of graded Shh signals into all-or-none distinctions in progenitor cell identity depends on crossrepressive interactions between selected pairs of class I and II proteins (Ericson et al., 1997; Briscoe et al., 2000; Sander et al., 2000; Muhr et al., 2001). In addition, most class I and II proteins have been shown to function directly as transcriptional repressors, through the recruitment of corepressors of the Gro/TLE class (Muhr et al., 2001). These findings have suggested a derepression model of neural patterning which invokes the idea that the patterning activities of individual class I or II proteins are achieved primarily through their ability to repress expression of complementary homeodomain proteins from specific progenitor domains. A central implication of this model is that homeodomain proteins direct progenitor cells to individual neuronal fates by suppressing alternative pathways of differentiation—a view that has strong parallels with proposed mechanisms of lineage restriction during lymphoid differentia-

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tion (Nutt et al., 1999; Rolink et al., 1999; Eberhard et al., 2000).

Much of the evidence that has led to this general outline of ventral neural patterning has emerged from an analysis of members of the *Nkx* gene family. Two closely related Nkx repressor proteins, Nkx2.2 and Nkx2.9, function as class II proteins that specify the identity of V3 neurons (Ericson et al., 1997; Briscoe et al., 1999, 2000). A more distantly related class II repressor protein, Nkx6.1, is expressed throughout the ventral third of the neural tube and when ectopically expressed, can direct motor neuron and V2 neuron fates (Briscoe et al., 2000; Sander et al., 2000). These gain-of-function studies are supported by an analysis of mice lacking *Nkx6.1* function, which exhibit a virtually complete failure in V2 interneuron generation (Sander et al., 2000). *Nkx6.1* null mice also show a reduction in motor neuron generation at rostral levels of the spinal cord, but at more caudal levels, motor neurons are formed in near-normal numbers (Sander et al., 2000). This observation reveals the existence of an Nkx6.1-independent program of spinal motor neuron generation, although the molecular basis of this alternative pathway is unclear.

A close relative of *Nkx6.1*, termed *Nkx6.2* (also known as *Nkx6B* or *Gtx*), has been identified (Komuro et al., 1993; Lee et al., 2001) and is expressed by neural progenitor cells (Cai et al., 1999). In its alias of Gtx, Nkx6.2 has been suggested to regulate myelin gene expression (Komuro et al., 1993), but its possible functions in neural patterning have not been examined. The identification of an *Nkx6* gene pair prompted us to address three poorly resolved aspects of ventral neural patterning. First, do closely related pairs of repressor homeodomain proteins serve distinct or redundant roles in ventral neural patterning? Second, are class I repressor proteins always complemented by a corresponding class II repressor, and if so, is Nkx6.2 one of the missing class II proteins? Third, to what extent is the generation of spinal motor neurons dependent on the activity of Nkx6 class proteins?

To address these issues, we mapped the profile of expression of Nkx6.2 and Nkx6.1 during neural tube development, and analyzed mouse *Nkx6* mutants to determine the respective contributions of these two genes to neural patterning. We show that Nkx6.2, like Nkx6.1, functions as a class II repressor homeodomain protein. Our analysis of *Nkx6* mutants further indicates that the duplication of an ancestral *Nkx6* gene has resulted in the expression of two proteins that exert markedly different levels of repressor activity in the ventral neural tube. This differential repressor activity of these two proteins appears to provide both a fail-safe mechanism during motor neuron generation and the potential for enhanced diversification of ventral interneuron subtypes. Moreover, we find that under conditions of reduced *Nkx6* gene dosage, ventral neuronal subtypes can be generated from progenitor cells that lack the class I or class II proteins normally required for their generation. This finding supports one of the central tenets of the derepression model of ventral neural patterning—that progenitor homeodomain proteins direct particular neuronal fates by actively suppressing cells from adopting alternative fates.

Results

Distinct Patterns of Nkx6.1 and Nkx6.2 Expression in Embryonic Spinal Cord

To examine the roles of *Nkx6* class genes in ventral neuronal specification, we compared the patterns of expression of Nkx6.2 and Nkx6.1 with that of other progenitor homeodomain proteins in the spinal cord of mouse and chick embryos. In the caudal neural tube of the mouse, the expression of Nkx6.2 was first detected at ~e8.5, in a broad ventral domain that largely coincided with that of Nkx6.1 (Figure 1A). Between e8.5 and e9.5, the expression of Nkx6.2 was lost from most Nkx6.1$^+$ cells in the ventral neural tube, although expression persisted in a narrow stripe of cells just dorsal to the limit of Nkx6.1 expression (Figures 1B and 1C). At e10.0–e10.5, virtually all Nkx6.2$^+$ cells coexpressed Dbx2 (Figure 1E), and the ventral limit of expression of both Nkx6.2 and Dbx2 coincided with the dorsal limit of Nkx6.1 expression at the p1/p2 domain boundary (Figures 1D and 1E). Nkx6.2 was expressed predominantly within the p1 domain, but scattered Nkx6.2$^+$ cells were detected within the p0 domain—the domain of expression of Pax7$^-$, Dbx1$^+$ cells (Figure 1F). Within the p0 domain, however, individual Nkx6.2$^+$ cells did not coexpress Dbx1, although they did express Dbx2 (Figures 1E–1G). Thus, the scattered Nkx6.2$^+$ cells found at the dorsoventral level of the p0 domain exhibit a p1, rather than p0, progenitor cell identity. Studies in chick have similarly shown that p0 and p1 progenitors are interspersed in the most dorsal domain of the ventral neural tube (Pierani et al., 1999).

In the chick neural tube, as in the mouse, *Nkx6.1* and *Nkx6.2* are initially coexpressed in a broad ventral domain (Cai et al., 1999; data not shown). But in contrast to the mouse, *Nkx6.2* expression persists in ventral progenitor cells, with the consequence that the expression of *Nkx6.2* and *Nkx6.1* also overlaps at later developmental stages (Figures 1H and 1I). Nevertheless, expression of chick *Nkx6.2* is also detected in a thin stripe of cells dorsal to the limit of *Nkx6.1* expression, within the p1 domain (Figure 1H). Thus, in both species, p1 progenitors coexpress Nkx6.2 and Dbx2 and exclude Nkx6.1.

Nkx6.2 Regulates V0 and V1 Interneuron Fates by Repression of Dbx1 Expression

The establishment and maintenance of progenitor cell domains in the ventral neural tube have been proposed to depend on mutual repressive interactions between complementary pairs of class I and II homeodomain proteins (Briscoe et al., 2000; Muhr et al., 2001). But class II proteins have been identified for only two of the five known progenitor domain boundaries (the p1/p2 and pMN/p3 boundaries) (Ericson et al., 1997; Briscoe et al., 1999, 2000; Sander et al., 2000). The mutually exclusive pattern of expression of Nkx6.2 and Dbx1 within p1 and p0 progenitors led us to consider whether Nkx6.2 might function as a class II protein that represses Dbx1 expression, and thus help to establish the identity of p1 progenitor cells and the fate of their En1$^+$ V1 neuronal progeny.

To test this idea, we analyzed the profile of expression of class I and II homeodomain proteins in *Nkx6.2* mutant

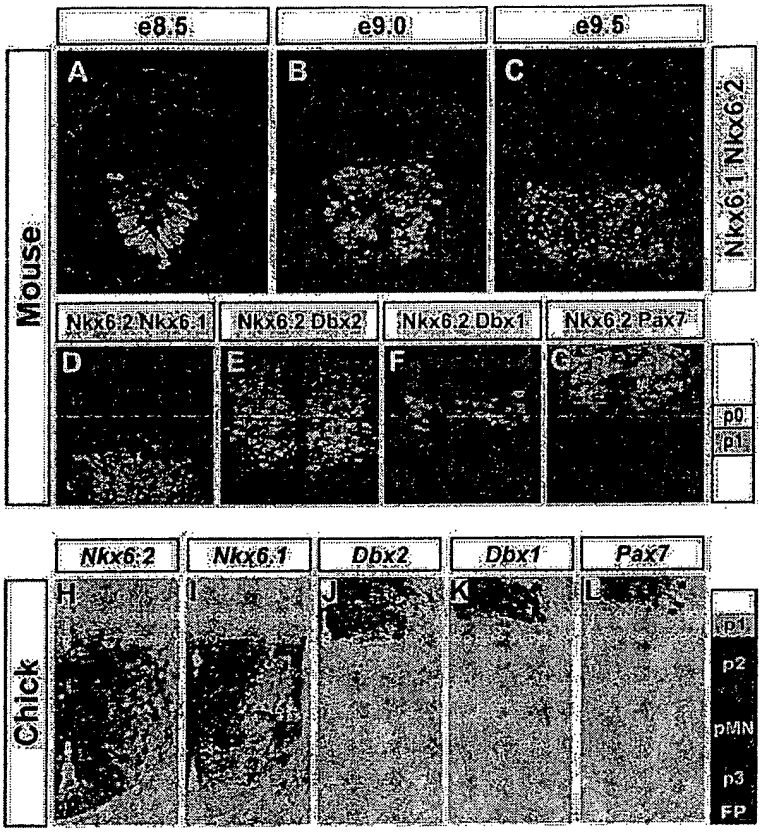

Figure 1. Expression of Nkx6.2 and Nkx6.1 in Developing Mouse and Chick Spinal Cord

(A) At e8.5, Nkx6.2 and Nkx6.1 are expressed in a broad ventral domain of the mouse neural tube. (B) At e9.0, Nkx6.2 expression is largely confined to a narrow domain immediately dorsal to the domain of Nkx6.1 expression. A few scattered cells that coexpress Nkx6.2 and Nkx6.1 are detected in more ventral positions at this stage. (C) At e9.5, Nkx6.2 is expressed in a narrow domain, dorsal to the Nkx6.1 boundary.

(D–G) Comparative patterns of expression of Nkx6.2, Nkx6.1, Dbx2, Dbx1, and Pax7 in the intermediate region of e10.5 mouse spinal cord. (E) Virtually all Nkx6.2$^+$ cells coexpress Dbx2, but since the level of Dbx2 expression in individual p1 progenitors varies, some cells appear reddish rather than bright yellow.

(H–L) Expression pattern of *Nkx6.2*, *Nkx6.1*, *Dbx2*, *Dbx1*, and *Pax7* in HH stage 20 chick spinal cord. Panels on right indicate progenitor domains, defined according to Briscoe et al. (2000).

embryos. We inactivated the mouse *Nkx6.2* gene by homologous recombination in embryonic stem (ES) cells. A targeted *Nkx6.2* allele (*Nkx6.2^{tlz}*) was generated by replacing the coding sequence of *Nkx6.2* with a *tauLacZ* cassette (Figure 2A). In the spinal cord of *Nkx6.2$^{+/tlz}$* embryos analyzed at e10.5, expression of LacZ and Nkx6.2 coincided within the p1 progenitor domain (see Figures 2E and 2F). In *Nkx6.2$^{tlz/tlz}$* embryos, the location of LacZ$^+$ cells was also similar to that in *Nkx6.2$^{+/tlz}$* embryos (Figures 2F and 2G), but Nkx6.2 protein was not detected (Figure 2G). These data provide evidence that the *Nkx6.2^{tlz}* allele generates a null mutation, and that disruption of the *Nkx6.2* locus does not perturb the normal spatial pattern of expression of this gene.

We did observe, however, that the level of LacZ expression was markedly elevated in *Nkx6.2$^{tlz/tlz}$*, when compared with *Nkx6.2$^{+/tlz}$*, embryos (Figures 2B–2D). An elevation in level of expression of the residual 5′ *Nkx6.2* transcript was also detected in *Nkx6.2$^{tlz/tlz}$* embryos (Figures 2H–2J). These observations provide evidence that Nkx6.2 negatively regulates its own expression level within p1 progenitor cells.

We next analyzed the pattern of expression of class I and II homeodomain proteins in the spinal cord and caudal hindbrain of *Nkx6.2$^{tlz/tlz}$* embryos. The domains of expression of the class II proteins Nkx2.2 and Nkx6.1, and of the class I proteins Pax7, Dbx2, Irx3, and Pax6, were similar in *Nkx6.2 $^{tlz/tlz}$*, *Nkx6.2$^{+/tlz}$*, and wild-type embryos (Figures 3B–3D and 3G–3I; data not shown). In addition, normal patterns of expression of Dbx2 and Nkx6.1 were detected at the p1/p2 domain boundary (data not shown), showing that establishment of the p1 progenitor domain does not require Nkx6.2 function. However, the level of Dbx2 expression in p1 domain progenitors was increased ~2-fold in Nkx6.2$^{tlz/tlz}$ mutants (Figures 2K–2M), indicating that Nkx6.2 normally limits the level of Dbx2 expression in this domain.

We also detected a marked change in the pattern of expression of the p0 progenitor cell marker Dbx1 in *Nkx6.2$^{tlz/tlz}$* embryos. At caudal hindbrain levels, the number of ventral Dbx1$^+$ progenitor cells increased 1.7- fold (Figure 3F), and the domain of Dbx1$^+$ cells expanded ventrally, extending through the p1 domain to the dorsal limit of Nkx6.1 expression (Figure 3H). Moreover, in *Nkx6.2$^{tlz/tlz}$* embryos, all of the ectopic Dbx1$^+$ cells found within the p1 domain coexpressed LacZ (Figure 3J). Thus, many progenitors within the p1 domain initiate Dbx1 expression in the absence of Nkx6.2 function. Nevertheless in *Nkx6.2$^{tlz/tlz}$* embryos, numerous LacZ$^+$ progenitors still lacked Dbx1 expression (Figure 3J), implying the existence of an Nkx6.2-independent means of excluding Dbx1 expression from p1 progenitors. The ventral expansion of Dbx1 was most prominent at caudal hindbrain and cervical spinal levels of the neural tube, but a similar, albeit less marked, expansion of Dbx1 expression was detected at caudal spinal levels (data not shown; see Figure 6). Taken together, these data imply that within p1 domain progenitors, Nkx6.2 functions as a weak repressor of Dbx2 expression and a more potent repressor of Dbx1 expression.

We next analyzed the generation of interneuron sub-

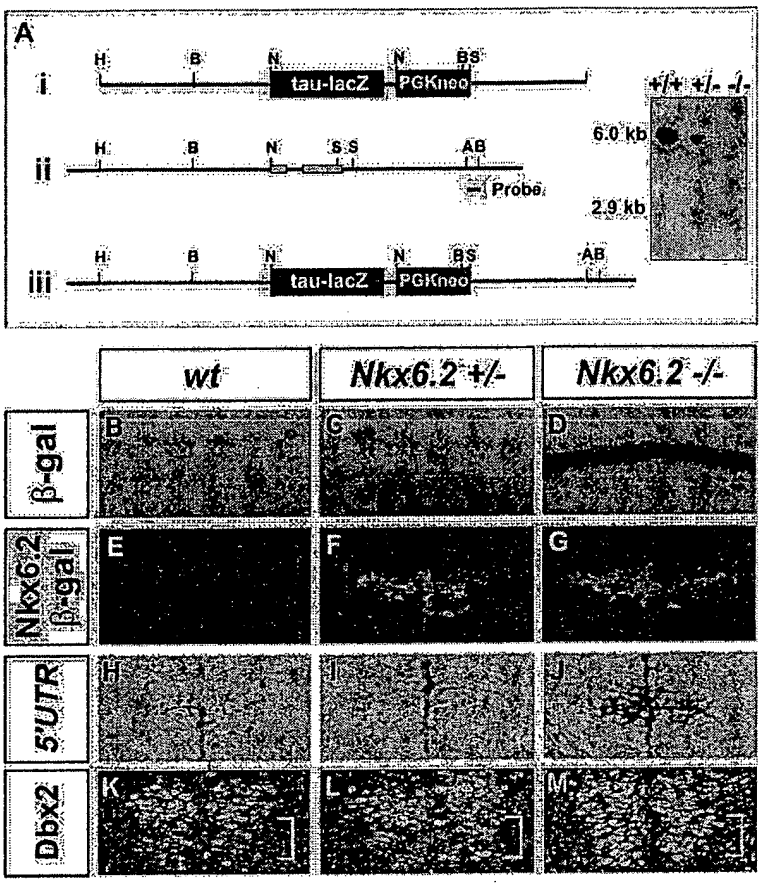

Figure 2. Elevation in *Nkx6.2* and Dbx2 Expression in p1 Domain Cells in *Nkx6.2* Mouse Mutants

(A) Diagram of the targeting construct (i) used to replace the coding sequence of *Nkx6.2* (ii) with a tau-lacZ PGK-neo cassette (iii). Red bar indicates region used as probe in genotyping.

(B–D) Sagital view of e10.5 spinal cord showing LacZ expression, detected by X-gal staining, in wild-type (*wt*), (B) *Nkx6.2*$^{+/tlz}$ (C), and *Nkx6.2*$^{tlz/tlz}$ (D) embryos.

(E–G) Nkx6.2 and LacZ expression in the p1 domain of *wt* (E), *Nkx6.2*$^{+/tlz}$ (F), and *Nkx6.2*$^{tlz/tlz}$ (G) embryos at e10.5.

(H–J) In situ hybridization with a 5′UTR probe shows that expression of *Nkx6.2* is elevated in the p1 domain of *Nkx6.2*$^{tlz/tlz}$ embryos (J), compared with *wt* (H) or *Nkx6.2*$^{+/tlz}$ (I) embryos.

(K–M) Expression of Dbx2 is upregulated ~2-fold in cells within the p1 domain (yellow bracket) in *Nkx6.2*$^{tlz/tlz}$ embryos (M), compared with *wt* (K) or *Nkx6.2*$^{+/tlz}$ (L) embryos. Abbreviations in (A): H = HindIII, B = BamHI, N = NcoI, S = SphI, A =AccI.

types in the ventral neural tube. In wild-type embryos, Dbx1$^+$, Dbx2$^+$, Nkx6.2$^-$ p0 progenitors generate Evx1/2$^+$ V0 neurons (Pierani et al., 1999, 2001); Nkx6.2$^+$, Dbx1$^-$, Dbx2$^+$ p1 progenitors give rise to En1$^+$ V1 neurons (Burrill et al., 1997; Ericson et al., 1997); and Nkx6.1$^+$, Irx3$^+$, p2 progenitors give rise to Chx10$^+$ V2 neurons (Ericson et al., 1997; Briscoe et al., 2000). Dbx1 activity in p0 progenitors is required to promote V0 and suppress V1 neuronal fates (Pierani et al., 2001). The ventral expansion in Dbx1 expression in *Nkx6.2*$^{tlz/tlz}$ embryos therefore led us to examine whether the loss of Nkx6.2 function leads progenitor cells within the p1 domain to adopt a V0 rather than V1 neuronal fate.

In the caudal hindbrain of *Nkx6.2* $^{tlz/tlz}$ embryos examined at e10.5, we detected a ~2-fold increase in the number of Evx1/2$^+$ V0 neurons, and the domain of V0 neuronal generation expanded ventrally to the normal position of the p1 domain (Figure 3N). Consistent with this, many Evx1/2$^+$ neurons coexpressed LacZ (Figure 3P), showing directly that some V0 neurons derive from p1 progenitors in the absence of Nkx6.2 function. Conversely, the total number of En1$^+$ V1 neurons generated in *Nkx6.2*$^{tlz/tlz}$ embryos was reduced by ~50% (Figure 3Q). The dorsoventral position of generation of the remaining En1$^+$ V1 neurons was similar in *Nkx6.2*$^{tlz/tlz}$ embryos (Figure 3N), and these neurons expressed LacZ (Figure 3O), showing directly that Nkx6.2$^+$, Dbx2$^+$ p1 progenitor cells generate V1 neurons. The total number of neurons generated from p1 domain progenitors, defined by Cyn1, TuJ1, and Lim1/2 expression, was similar in *Nkx6.2*$^{tlz/tlz}$ and *Nkx6.2*$^{+/tlz}$ embryos examined at e10.5 (data not shown). In addition, the number of TUNEL$^+$ cells was similar in *Nkx6.2*$^{tlz/tlz}$ and *Nkx6.2*$^{+/tlz}$ embryos (data not shown). Chx10$^+$ V2 neurons and HB9$^+$, Isl1/2$^+$ motor neurons were present in normal numbers and positions in *Nkx6.2*$^{tlz/tlz}$ embryos (Figure 5; data not shown). Together, these findings show that the activity of Nkx6.2 within p1 progenitors promotes V1 neuronal generation and helps to suppress the generation of V0 neurons, a finding consistent with the proposed role of Nkx6.2 in repressing Dbx1 expression from p1 progenitors.

Repression of Nkx6.2 by Nkx6.1 Underlies *Nkx6* Gene Redundancy in Spinal Motor Neuron Generation

We next addressed the respective contributions of *Nkx6.1* and *Nkx6.2* to motor neuron and V2 neuron generation. In the ventral neural tube, p2 and pMN progenitors express Nkx6.1 and give rise to V2 neurons and motor neurons, respectively. Ectopic expression of Nkx6.1 is sufficient to induce motor neurons and V2 interneurons in dorsal regions of the neural tube, and in *Nkx6.1* mutant mice, V2 neurons are eliminated (Briscoe et al., 2000; Sander et al., 2000). Nevertheless, there is only a partial reduction in motor neuron generation in *Nkx6.1* mutants (Sander et al., 2000), revealing the existence of an *Nkx6.1*-independent pathway of motor neuron generation. Nkx6.2 does not normally contribute to motor neuron specification in the mouse since its expression is

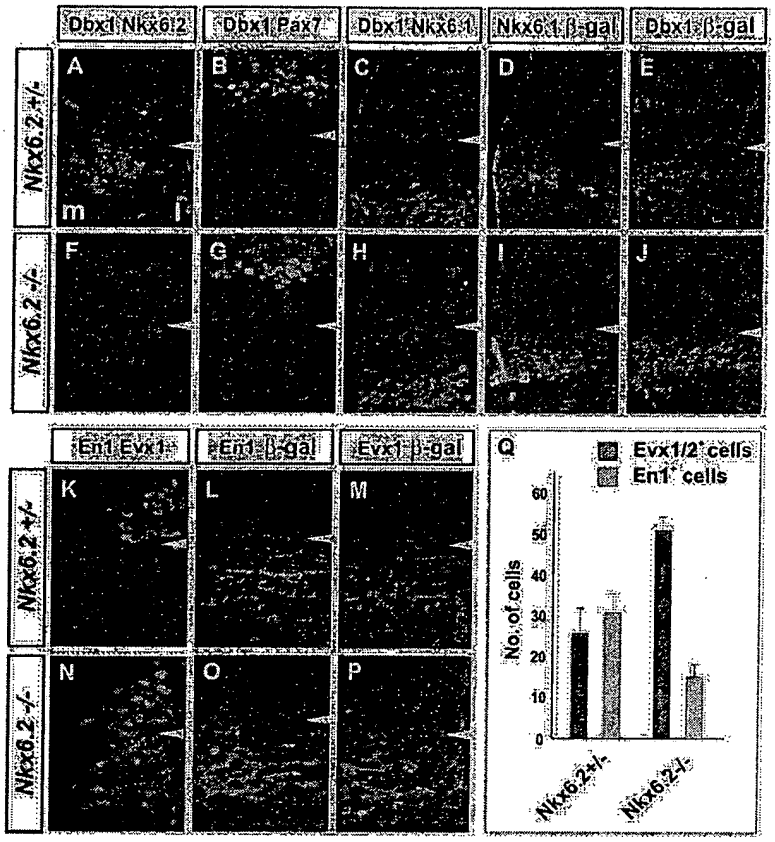

Figure 3. A Partial Switch from V1 to V0 Neuronal Fate in *Nkx6.2* Mutant Mice

(A–E) Expression of Nkx6.2 (A), Nkx6.1 (C and D), Dbx1 (B, C, and E), and Pax7 (B) appears normal at caudal hindbrain levels of e10.5 *Nkx6.2*$^{+/tlz}$ embryos. The expression of Nkx6.1 (D) and Dbx1 (E) abuts the ventral and dorsal boundaries of *LacZ* expression.

(F–J) In e10.5 *Nkx6.2*$^{tlz/tlz}$ embryos, expression of Nkx6.1 (H and I) and Pax7 (G) is unchanged, but expression of Dbx1 (F, G, and H) is expanded ventrally into the p1 domain. Many ventral ectopic Dbx1$^+$ cells in *Nkx6.2*$^{tlz/tlz}$ embryos express LacZ (J).

(K–M) Evx1/2$^+$ V0 neurons are generated dorsal to En1$^+$ V1 neurons (K) and *LacZ*$^+$ cells (M) in *Nkx6.2*$^{+/tlz}$ embryos. En1$^+$ neurons express LacZ in *Nkx6.2*$^{+/tlz}$ (L) and *Nkx6.2*$^{tlz/tlz}$ (O) embryos.

(N–P) Evx1/2$^+$ V0 neurons are generated in increased numbers and at ectopic ventral positions in the caudal hindbrain of *Nkx6.2*$^{tlz/tlz}$ embryos. (N) The number of En1$^+$ V1 neurons is reduced and the remaining En1$^+$ neurons are intermingled with ectopic Evx1/2$^+$ cells. (P) Many Evx1/2$^+$ neurons in *Nkx6.2*$^{tlz/tlz}$ embryos coexpress LacZ.

(Q) Quantitation of Evx1/2$^+$ V0, and En1$^+$ V1 neurons at the caudal hindbrain of *Nkx6.2*$^{+/tlz}$ and *Nkx6.2*$^{tlz/tlz}$ embryos at e10.5. Counts from 12 sections, mean ± SD. In panels (A)–(P), the white arrowhead indicates the p0/p1 boundary. The mouse *Nkx6.2* gene has also been inactivated by Cai et al. (2001), but a similar V1 to V0 interneuron fate change was not noted.

extinguished from ventral progenitors well before the appearance of post-mitotic motor neurons (Figures 1A–1C), and there is no change in the number of motor neurons generated in *Nkx6.2*$^{tlz/tlz}$ embryos (see Figure 5G).

Three lines of evidence, however, led us to consider a cryptic role for Nkx6.2 in motor neuron generation. First, Nkx6.2 and Dbx2 share the same ventral limit of expression at the p1/p2 domain boundary, and the expression of Dbx2 is repressed by Nkx6.1 (Briscoe et al., 2000; Sander et al., 2000). Second, Nkx6.2 negatively regulates its own expression level within p1 domain progenitors (Figures 2D, 2G, and 2J). Third, Nkx6.1 and Nkx6.2 possess similar Gro/TLE recruitment activities and DNA target site binding specificities (Muhr et al., 2001). We reasoned therefore that under conditions in which Nkx6.1 activity is reduced or eliminated, Nkx6.2 expression might be derepressed in p2 and pMN progenitors.

In support of this idea, in *Nkx6.1*$^{+/-}$ embryos examined at e10.5, we detected a marked increase in the number of Nkx6.2$^+$ cells within the p2 and pMN domains (Figure 4B). And in *Nkx6.1*$^{-/-}$ embryos, expression of Nkx6.2 was detected in virtually all progenitor cells within the p2 and pMN domains (Figure 4C). Indeed, in *Nkx6.1*$^{-/-}$ embryos, the level of Nkx6.2 expression in the nuclei of progenitor cells within the p2 and pMN domains was 1.9-fold greater than that in progenitor cells located within the p1 domain (Figure 4C; data not shown). Together, these data show that Nkx6.1 activity normally represses Nkx6.2 expression from p2 and pMN progenitors in the mouse embryo.

In turn, these findings raised the possibility that in *Nkx6.1*$^{-/-}$ embryos, the derepression of Nkx6.2 expression substitutes for the loss of Nkx6.1 during motor neuron generation. If this is the case, Nkx6.2 would be predicted to mimic the ability of Nkx6.1 to induce motor neurons in vivo. Expression of chick or mouse *Nkx6.2* in the neural tube of HH stage 10–12 chick embryos repressed Dbx2 and Dbx1 expression (Figures 4D–4F), and induced ectopic motor neuron differentiation (Figures 4G–4I and 4L–4N) with an efficacy similar to that of *Nkx6.1* (Briscoe et al., 2000). These data show that Nkx6.2 can induce ectopic motor neurons when expressed at high levels in the dorsal neural tube, supporting the idea that both Nkx6 proteins can exert similar patterning activities in vivo (Figures 4D–4O; Briscoe et al., 2000). In addition, misexpression of Nkx6.2 in the p0 and p1 progenitor domains suppressed the generation of Evx1/2$^+$ V0 and En1$^+$ V1 neurons and promoted the generation of Chx10$^+$ V2 neurons (Figures 4J, 4K, 4O, and 4P). Thus, a high level of expression of Nkx6.2 is not compatible with the generation of either V0 or V1 neurons (Figures 4O and 4P).

Based on these findings, we examined whether Nkx6.2 has a role in motor neuron generation in *Nkx6.1* mutant mice by testing the impact of removing Nkx6.2 as well as Nkx6.1 on the generation of spinal motor neurons. In *Nkx6.2*$^{tlz/tlz}$ embryos, there was no change in the number of motor neurons generated at any level of the spinal cord or hindbrain (Figures 5G, 5N, and 5O; data not shown). In *Nkx6.1*$^{-/-}$ mutants, the number of spinal motor neurons was reduced by ~60% at cervical

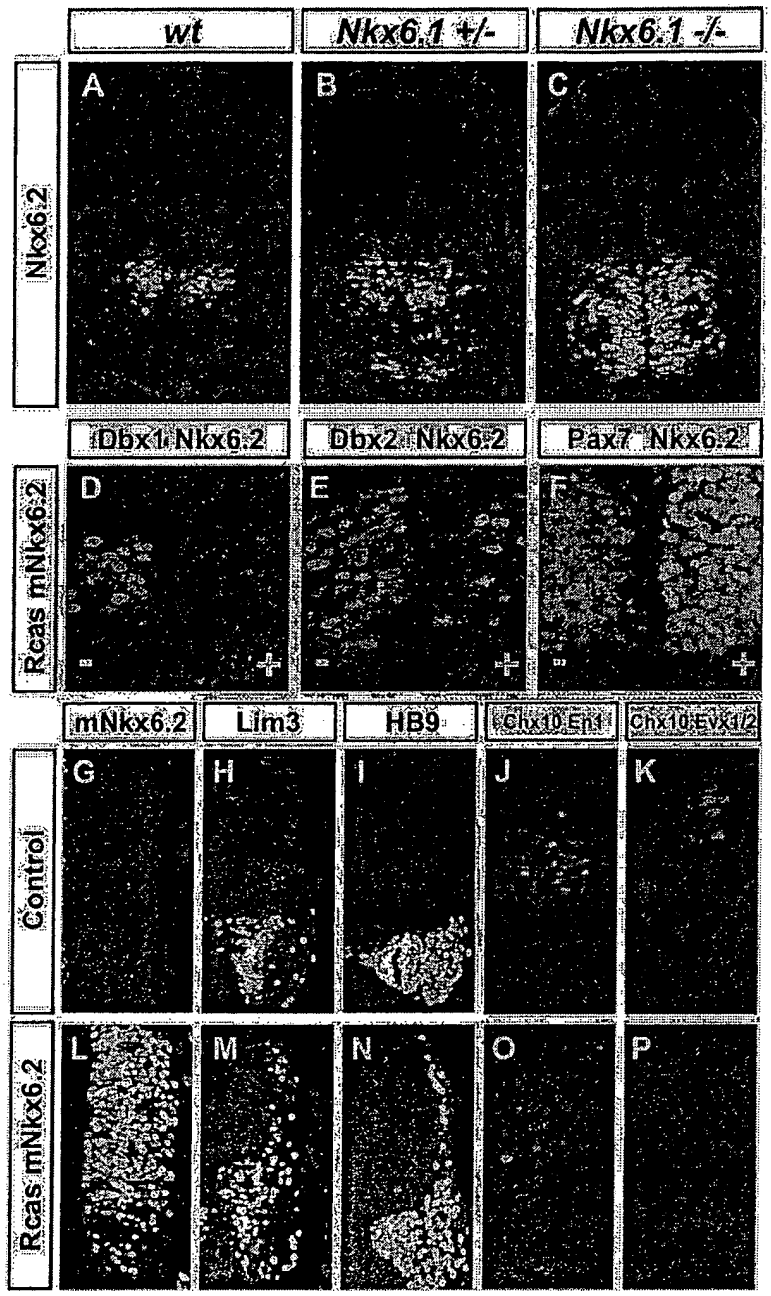

Figure 4. Deregulated Expression of Nkx6.2 in *Nkx6.1* Mutant Mice, and Similar Patterning Activities of Nkx6 Proteins in Chick Neural Tube

(A) In e10.5 *wt* embryos, Nkx6.2 expression is confined to the p1 progenitor domain. (B) In *Nkx6.1*$^{+/-}$ embryos, scattered Nkx6.2^{+} cells are detected in the p2, pMN, and p3 domains. (C) In Nkx6.1$^{-/-}$ embryos, Nkx6.2 is expressed in most progenitors in the p2, pMN, and p3 domains.

(D–F) Misexpression of Nkx6.2 at high levels represses the expression of Dbx1 (D) and Dbx2 (E), but not Pax7 (F).

(G–P) Expression of Nkx6.2 in dorsal positions of the chick neural tube result in ectopic dorsal generation of motor neurons, as indicated by ectopic induction of Lim3 and HB9 expression (G–I and L–N). Forced expression of Nkx6.2 at high levels in the p0 and p1 progenitor domains promotes the ectopic generation of Chx10^{+} V2 neurons (J, K, O, and P) and suppresses Evx1/2^{+} V0 (K and P) and En1^{+} V1 (J and O) neurons.

levels, but by only 25% at lumbar levels (Figures 5H, 5N, and 5O; Sander et al., 2000). In *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{+/tlz}$ embryos, motor neuron generation was reduced to ~25% of controls at both cervical and lumbar levels (Figures 5I, 5N, and 5O; data not shown). In *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{tlz/tlz}$ embryos, the generation of motor neurons was reduced to <10% of wild-type numbers, at all levels of the spinal cord (Figure 5J). In these *Nkx6* double mutant embryos, residual motor neurons were detected at e10.0, and no further increase in motor neuron number was evident at e12 (Figures 5M and 5P; data not shown). Since there was no increase in apoptotic cell death in the ventral neural tube over this period (data not shown), we infer that the few spinal motor neurons present in *Nkx6* double mutants are generated prior to e10. Together, these findings demonstrate that Nkx6.2 substitutes for the loss of Nkx6.1 in spinal motor neuron generation, and reveal a link between *Nkx6* gene dosage and the incidence of motor neuron generation.

A Dissociation in Neuronal Fate and Progenitor Cell Identity in *Nkx6* Mutant Mice

We next examined whether a reduction in *Nkx6* gene dosage results in ectopic Dbx protein expression and V1 and V0 neuron generation in the p2 and pMN domains of the ventral spinal cord.

En1^{+} V1 neurons are normally generated from Dbx2^{+}, Dbx1^{-} p1 progenitor cells, and we therefore analyzed the relationship between Dbx2 expression and En1^{+} V1 neuronal generation in *Nkx6.1* and *Nkx6.2* compound

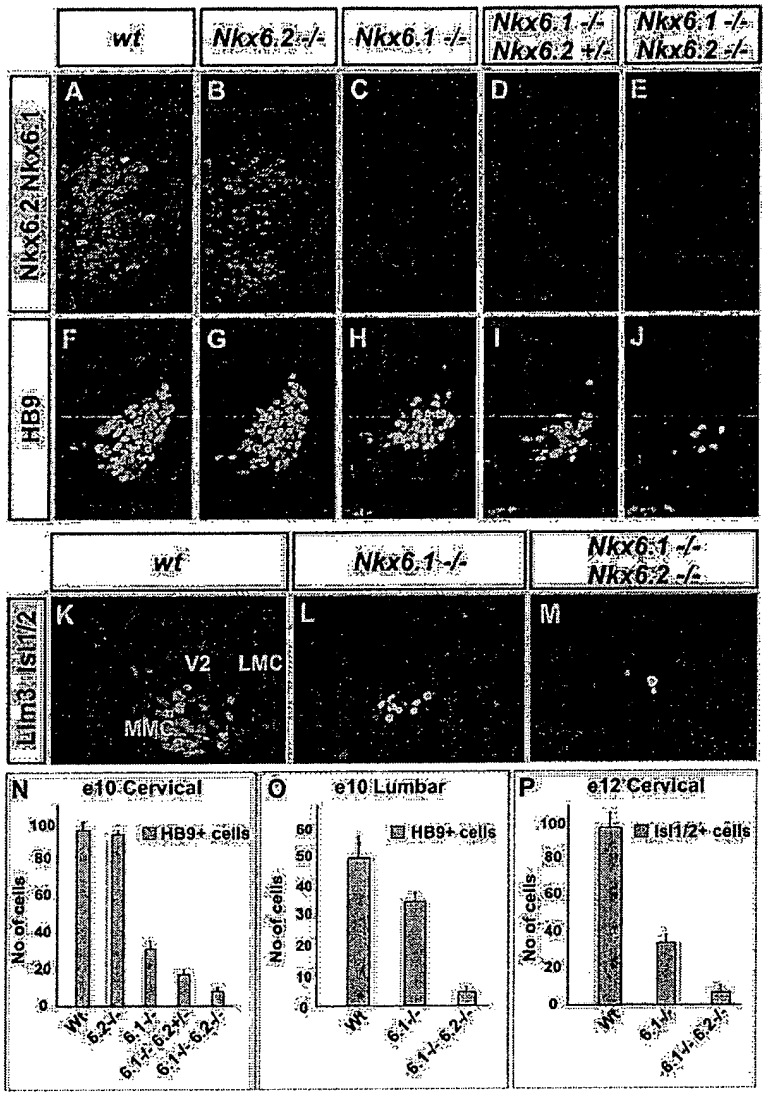

Figure 5. The Deregulated Expression of Nkx6.2 Underlies Motor Neuron Generation in *Nkx6.1* Mutants

(A) In e10.5 *wt* embryos, Nkx6.2 expression is confined to the p1 domain and Nkx6.1 is expressed in the p2, pMN, and p3 domains.

(B) No change in the expression of Nkx6.1 is detected in *Nkx6.2* $^{tlz/tlz}$ embryos.

(C and D) In *Nkx6.1* $^{-/-}$ and *Nkx6.1* $^{-/-}$; *Nkx6.2* $^{+/tlz}$ embryos, Nkx6.2 expression is derepressed in the p2, pMN, and p3 domains.

(E) No expression of Nkx6.2 or Nkx6.1 protein is detected in *Nkx6.1* $^{-/-}$; *Nkx6.2* $^{tlz/tlz}$ embryos.

(F and G) HB9$^+$, Isl1/2$^+$ motor neurons are generated in normal numbers in *Nkx6.2* $^{tlz/tlz}$ embryos. The number of motor neurons is reduced by ~60% in *Nkx6.1* $^{-/-}$ embryos (H), by ~80% in *Nkx6.1* $^{-/-}$;*Nkx6.2* $^{+/tlz}$ embryos (I), and by >90% in *Nkx6.1* $^{-/-}$;*Nkx6.2* $^{tlz/tlz}$ at cervical levels of e10.5 spinal cord (J).

(K–M) At e12, the number of motor neurons of medial (MMC) (Isl1$^+$, Lim3$^+$) and lateral (LMC) (Isl1$^+$) subtype identity is reduced in similar proportions in *Nkx6.1* $^{-/-}$ and *Nkx6.1* $^{-/-}$; *Nkx6.2* $^{tlz/tlz}$ embryos. Lim3$^+$ V2 neurons are missing in *Nkx6.1* $^{-/-}$ embryos and *Nkx6.1* $^{-/-}$; *Nkx6.2* $^{tlz/tlz}$ embryos at this stage.

(N–P) Quantitation of HB9$^+$ and Isl1/2$^+$ motor neurons at cervical and lumbar levels in *wt*, *Nkx6.2*, and *Nkx6.1* single mutants and in *Nkx6.2; Nkx6.1* compound mutants at e10 and e12. Counts from 12 sections, mean ± SD.

mutants. As reported previously (Sander et al., 2000), in *Nkx6.1* $^{-/-}$ embryos examined at e10.5, ectopic ventral expression of Dbx2 was detected at high levels in the p2 and p3 domains, although cells in the pMN expressed only very low levels of Dbx2 (Figure 6H; see Sander et al., 2000). Moreover, in *Nkx6.1* $^{-/-}$ embryos, ectopic En1$^+$ neurons were generated in the p2 and pMN domains of the ventral neural tube (Figure 6R). In *Nkx6.1* $^{-/-}$; *Nkx6.2* $^{+/tlz}$ embryos, Dbx2 expression was detected at intermediate levels in the pMN domain (Figure 6I), and in *Nkx6.1* $^{-/-}$; *Nkx6.2* $^{tlz/tlz}$ double mutant embryos, Dbx2 was detected at uniformly high levels in the p2 and pMN domains (Figure 6J). Strikingly, in these *Nkx6.1* and *Nkx6.2* compound mutant backgrounds, and despite the enhanced ectopic expression of Dbx2, the number of ectopic ventral En1$^+$ V1 neurons was reduced rather than increased, when compared with the number generated in *Nkx6.1* single mutants (Figures 6R and 6T).

Since Evx1$^+$ V0 neurons are normally generated from Dbx1$^+$, Dbx2$^+$ p0 progenitors, we examined whether the reduction in ectopic ventral En1$^+$ V1 neuron generation at low *Nkx6* gene dosage might reflect a change in the pattern of expression of Dbx1, and the ectopic generation of V0 neurons. Consistent with this idea, in *Nkx6.1* $^{-/-}$; *Nkx6.2* $^{tlz/tlz}$ mutants, scattered Dbx1$^+$ cells were detected in the p2, pMN, and p3 domains (Figure 6O), and ectopic ventral Evx1/2$^+$ V0 neurons were detected throughout the ventral neural tube (Figures 6T and 6Z). Thus, in *Nkx6* double mutants, the loss of V1 neurons is associated with the ectopic ventral expression of Dbx1 and the generation of ectopic V0 neurons.

But in *Nkx6.1* single and *Nkx6.1* $^{-/-}$; *Nkx6.2* $^{+/tlz}$ compound mutant backgrounds, the normal link between expression of Dbx1 in progenitor cells and the generation of Evx1/2$^+$ V0 neurons was severed. In both these *Nkx6* compound mutants backgrounds, the domain of expression of Dbx1 was unchanged (Figures 6M and 6N): a result that can be accounted for by the maintained expression of Nkx6.2 within the p1 domain, and the deregulated expression of Nkx6.2 within the p2 and pMN domains. Nevertheless, Evx1/2$^+$ V0 neurons were generated from progenitor cells in the position of p2 and pMN domains (Figures 6R, 6S, 6X, and 6Y).

We next considered whether these ectopic V0 neurons were generated from the position of the p2 and pMN domains, or whether they simply migrated ventrally

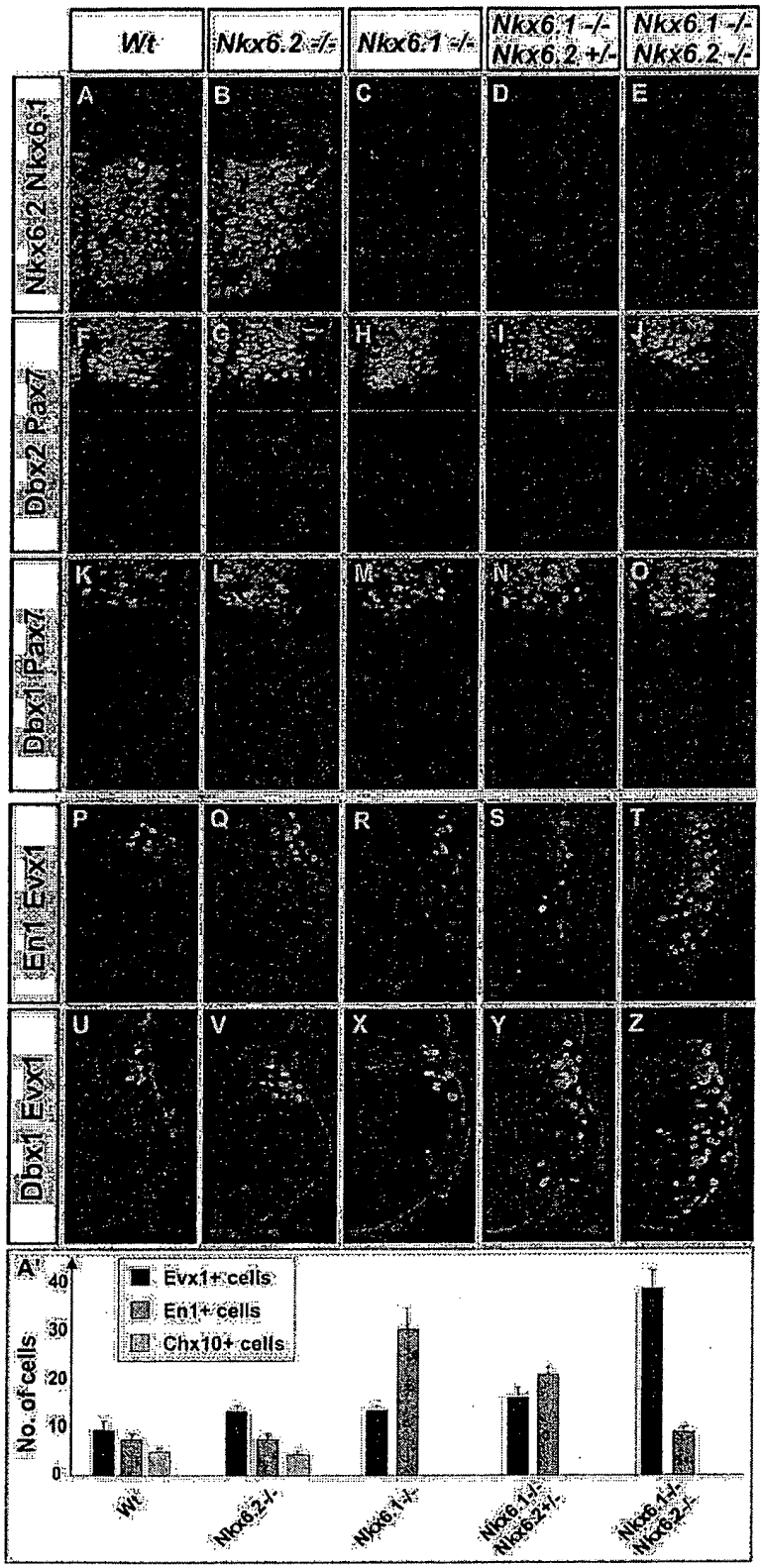

Figure 6. Changes in Class I Protein Expression and Ventral Interneuron Generation in *Nkx6* Mutants

(A–E) Expression of Nkx6.1 and Nkx6.2 in the spinal cord in different *Nkx6* mutant backgrounds at e10.5.

(F–J) Spatial patterns of Pax7 and Dbx2 expression in different *Nkx6* mutant backgrounds. Note that the level of Dbx2 expression in the pMN domain of Nkx6.1$^{-/-}$; Nkx6.2$^{+/tlz}$ is very low, implying the existence of a pMN domain-restricted gene that has the capacity to repress Dbx2 expression. Recent studies have provided evidence that the bHLH protein Olig2 possesses these properties (Novitch et al., 2001).

(K–O) Spatial patterns of expression of Pax7 and Dbx1 in different *Nkx6* mutant backgrounds.

(P–T) Spatial patterns of generation of Evx1/2^{+} V0 neurons and En1^{+} V1 neurons in different *Nkx6* mutant backgrounds. (Q) The generation of V0 neurons expands ventrally into the p1 domain in *Nkx6.2$^{tlz/tlz}$* mutants at caudal spinal levels. (R and A') The number of En1^{+} V1 neurons increases ~3-fold in the ventral spinal cord of *Nkx6.1$^{-/-}$* mutants, and ectopic Evx1/2^{+} cells are detected in position of the pMN domain in these mice (see also Sander et al., 2000). (S, T, and A') There is a progressive increase in Evx1/2^{+} V0 neurons and a loss of En1^{+} V1 neurons in the ventral spinal cord of *Nkx6.1$^{-/-}$;Nkx6.2$^{+/tlz}$* and *Nkx6.1$^{-/-}$;Nkx6.2$^{tlz/tlz}$* embryos. (U,V, and Z) The generation of Evx1/2^{+} V0 neurons correlates with the pattern of expression of Dbx1 in progenitors in *wt*, *Nkx6.2$^{tlz/tlz}$*, and *Nkx6.1$^{-/-}$;Nkx6.2$^{tlz/tlz}$* mutant backgrounds. Note that only the most lateral progenitor cells express Dbx1 in Nkx6.1$^{-/-}$; Nkx6.2$^{tlz/tlz}$ embryos, suggesting that expression of Dbx1 in more medially positioned progenitors is repressed by an as yet undefined gene. (X and Y) Ectopic ventral Evx1^{+} V0 neurons derive from Dbx1^{-} progenitors in *Nkx6.1$^{-/-}$* and *Nkx6.1$^{-/-}$;Nkx6.2$^{+/tlz}$* mutant embryos. Chx10^{+} V2 neurons are generated at normal numbers in *Nkx6.2$^{tlz/tlz}$* mutants, but are missing at spinal cord levels in *Nkx6.1$^{-/-}$*, *Nkx6.1$^{-/-}$;Nkx6.2$^{+/tlz}$*, and *Nkx6.1$^{-/-}$;Nkx6.2$^{tlz/tlz}$* mutants (A'; Figure 5, see Sander et al., 2000).

from a more dorsal position of origin. Ectopic ventral Evx1/2^{+} V0 neurons were detected as early as e10.0 (Figure 7B), and many of them coexpressed LacZ (Figures 7C and 7D), providing evidence that many of these neurons derive from progenitor cells within the position of the p2 and pMN domains. The finding that Evx1/

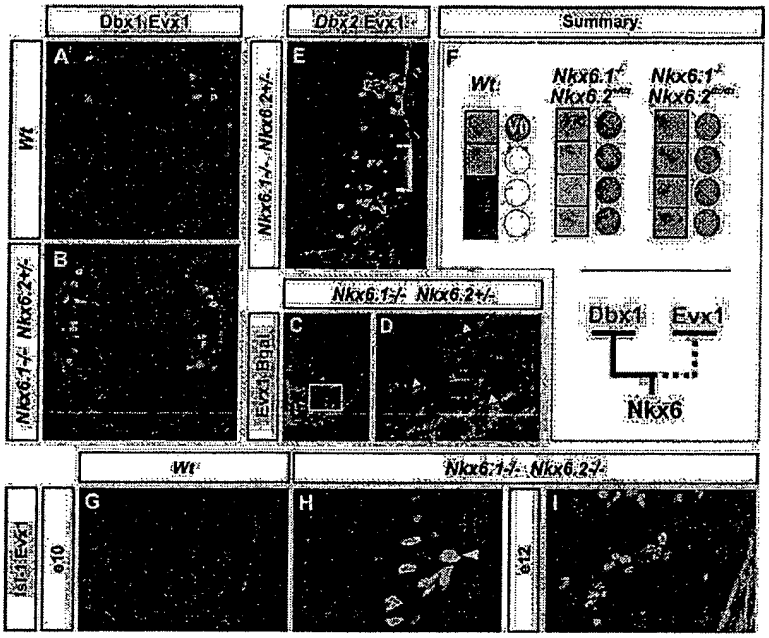

Figure 7. Dissociation of Dbx Expression and V0 Neuronal Fate in Mice with Reduced Nkx6 Protein Activity

(A) In e10.0 *wt* embryos, p0 progenitor cells express Dbx1 and generate Evx1/2$^+$ V0 neurons. (B) In e10.0 *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{+/tlz}$ embryos, there is no change in the domain of expression of Dbx1, but Evx1/2$^+$ V0 neurons are generated in lateral positions, along much of the ventral neural tube.

(C and D) In Nkx6.1$^{-/-}$; Nkx6.2$^{+/tlz}$ embryos examined at e10.0, many ectopic ventral Evx1/2$^+$ neurons express LacZ. Framed area in (C) is shown at high magnification in (D) and indicates Evx1/2$^+$ neurons that coexpress LacZ.

(E) Evx1/2$^+$ neurons located at the level of the pMN domain (bracket) derive from progenitors that express low or negligible levels of *Dbx2* mRNA.

(F) Summary of Dbx1 expression and V0 neuron generation in *wt*, *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{+/tlz}$, and *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{tlz/tlz}$ embryos. The dissociation of Dbx1 and Evx1/2 expression in *Nkx6.1*$^{-/-}$;*Nkx6.2*$^{+/tlz}$ embryo suggests that reduced Nkx6 repressor activity is sufficient to repress Dbx1, but insufficient to repress Evx1 expression.

2$^+$ V0 neurons are generated from the pMN domain in *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{+/tlz}$ embryos is especially significant since these progenitors express negligible levels of Dbx2 (Figures 7E and 8), arguing against the possibility that Dbx2 expression compensates for the absence of Dbx1 during ectopic V0 neuronal generation. These results therefore provide evidence that even though Dbx1 activity is normally required for the generation of V0 neurons (Pierani et al., 2001), under conditions in which *Nkx6* gene dosage is markedly reduced, V0 neurons can be generated from progenitor cells that lack Dbx1 expression.

Nevertheless, the pattern of ventral neurogenesis observed in *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{+/tlz}$ mutants indicated that residual Isl1/2$^+$, HB9$^+$ neurons and ectopic Evx1$^+$ neurons were each generated from progenitors located in the position of the pMN domain. This observation raised the question of whether these two neuronal populations are, in fact, distinct. Strikingly, we found that in this compound *Nkx6* mutant background, many of the resid-

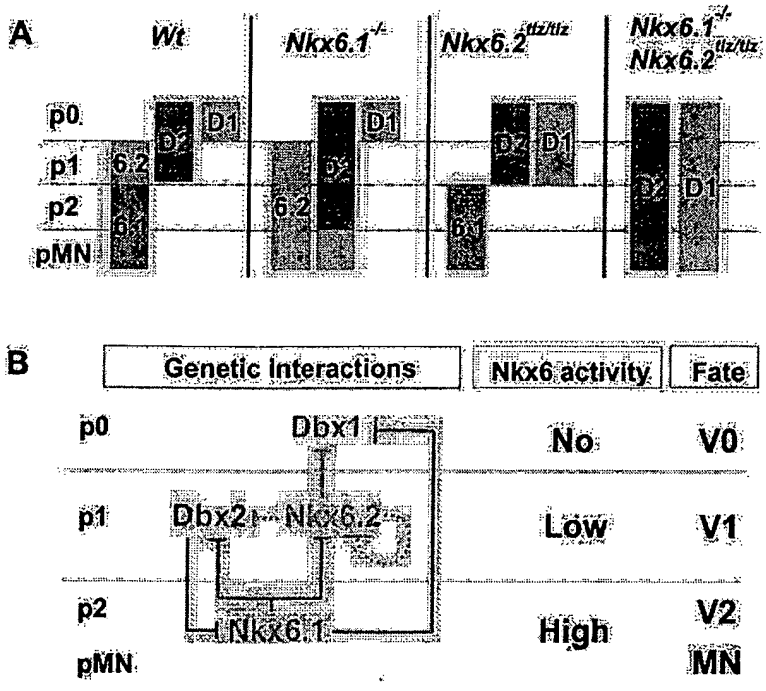

Figure 8. Genetic Interactions between Nkx6 and Dbx Proteins during the Assignment of Motor Neuron and Interneuron Fate in the Mouse Neural Tube

(A) Summary of domains of expression of Nkx6.1 (6.1), Nkx6.2 (6.2), Dbx1 (D1), and Dbx2 (D2) in the ventral neural tube of wild-type (wt) and different *Nkx6* mutant embryos.

(B) Regulatory interactions between Nkx and Dbx proteins in the ventral neural tube. These interactions result in different levels of Nkx6 protein activity in distinct ventral progenitor domains, and thus promote the generation of distinct neuronal subtypes.

For details see text.

ual Isl1/2$^+$, HB9$^+$ neurons transiently expressed Evx1 (Figures 7H and 7I). Thus, under conditions of reduced *Nkx6* gene dosage, progenitor cells at the position of the pMN domain initially generate neurons with a hybrid motor neuron/V0 neuron identity.

Discussion

The patterning of cell types in the ventral neural tube depends on the actions of a set of homeodomain proteins expressed by neural progenitor cells. Duplication of many of these genes has resulted in the overlapping neural expression of pairs of closely related homeodomain proteins, and raises the question of whether these proteins have distinct or redundant roles during ventral neurogenesis. We have used genetic approaches in mouse to examine the respective contributions of one such homeodomain protein pair, Nkx6.1 and Nkx6.2, in ventral neural patterning. Our results imply that the duplication of an ancestral *Nkx6* gene confers both redundant and distinct roles for Nkx6.1 and Nkx6.2 in ventral neuronal patterning. We discuss below how the specificity and efficacy of Nkx6-mediated transcriptional repression underlies the overlapping divergent patterning activities of the two proteins.

Redundant Activities of Nkx6 Proteins in Motor Neuron and V0 Neuron Generation

Our genetic studies in mice indicate that Nkx6.1 and Nkx6.2 have qualitatively similar activities in promoting the generation of motor neurons and in suppressing the generation of V0 neurons. How are these overlapping patterning activities achieved, given the distinct profiles of expression of these two genes?

Nkx6.1 has been shown to have a role in motor neuron generation (Sander et al., 2000), but the finding that large numbers of motor neurons are generated at caudal levels of the spinal cord in *Nkx6.1* mutant mice points to the existence of an Nkx6.1-independent pathway of motor neuron generation. At face value, Nkx6.2 would appear a poor candidate as a mediator of the Nkx6.1-independent pathway of motor neuron specification since it is not expressed by motor neuron progenitors, nor is motor neuron generation impaired in *Nkx6.2* mutant mice. Nevertheless, the activity of Nkx6.2 is responsible for the efficient generation of spinal motor neurons in *Nkx6.1* mutants. The basis of this redundant function resides in the derepression of Nkx6.2 expression in motor neuron progenitors in *Nkx6.1* mutant mice. Strikingly, Nkx6.2 is even derepressed in *Nkx6.1*$^{+/-}$ embryos, whereas there is no change in the patterns of expression of Dbx2 and other homeodomain proteins implicated in the repression of motor neuron generation. The propensity for Nkx6.2 derepression thus appears to establish a "fail-safe" mechanism that ensures that the net level of Nkx6 protein activity is maintained in motor neuron progenitors under conditions in which Nkx6.1 levels decrease. A similar "fail-safe" regulatory mechanism may operate with other Nkx protein pairs. During pharyngeal pouch development, for example, the loss of Nkx2.6 expression appears to be compensated for by the upregulation of Nkx2.5 (Tanaka et al., 2000).

The finding that Nkx6.2 is derepressed in the absence of Nkx6.1 function also offers a potential explanation for the divergent patterns of expression of Nkx6.2 in the ventral neural tube of mouse and chick embryos. We infer that the chick *Nkx6.2* gene is not subject to repression by Nkx6.1, permitting its persistent expression in p3, pMN, and p2 domain progenitor cells. Thus, in chick, the overlapping functions of Nkx6.1 and Nkx6.2 in motor neuron generation are associated with the coexpression of both genes by motor neuron progenitors, whereas in the mouse, Nkx6.2 activity is held in reserve, through its repression by Nkx6.1.

Nkx6.1 and Nkx6.2 also have an equivalent inhibitory influence on the generation of V0 neurons, albeit through activities exerted in different progenitor domains. In p1 progenitors, the repression of p0 identity and V0 neuron fate is accomplished by Nkx6.2. But ventral to the p1/p2 domain boundary, it is Nkx6.1 that prevents Dbx1 expression and V0 neuronal generation. Thus, Nkx6.1 is a potent repressor of Dbx1 expression, despite the fact that these two proteins lack a common progenitor domain boundary. The repression of genes that are normally positioned in spatially distinct domains has been observed with other class I and II proteins (Sander et al., 2000). This feature of neural patterning also parallels the activities of gap proteins in anteroposterior patterning of the *Drosophila* embryo, where the repressive activities of individual gap proteins are frequently exerted on target genes with which they lack a common boundary (Kraut and Levine, 1991; Stanojevic et al., 1991).

Distinct Functions of Nkx6.1 and Nkx6.2 in Ventral Interneuron Generation

We now turn to the question of how Nkx6.1 and Nkx6.2 can exert distinct roles in interneuron generation, given the similarities of the two proteins in DNA target site specificity (Jörgensen et al., 1999; Muhr et al., 2001), and their overlapping functions in the patterning of motor neurons and V0 neurons.

One factor that contributes to the opponent influence of Nkx6.1 and Nkx6.2 on the specification of V1 interneuron fate is a distinction in the dorsal limit of expression of the two proteins in the neural tube, presumably a reflection of differences in the regulation of expression of the two proteins by graded Shh signaling. Nkx6.1 expression stops at the p1/p2 domain boundary. And within the p2 domain, Nkx6.1 suppresses p1 progenitor identity through repression of Dbx2 and Nkx6.2 expression, in this way ensuring the generation of Chx10$^+$ V2 neurons. Nkx6.2, in contrast, occupies the p1 domain, where it is coexpressed with Dbx2. In p1 domain cells, Nkx6.2 promotes the generation of En1$^+$ V1 neurons by repressing the expression of Dbx1 and Evx1, determinants of V0 neuronal fate (Pierani et al., 2001; Moran-Rivard et al., 2001). Nevertheless, only a fraction of p1 progenitors initiate Dbx1 expression and acquire V0 neuron fate in the absence of Nkx6.2 function, raising the possibility that Dbx2 may also have a role in repressing Dbx1 expression within p1 progenitors (see Pierani et al., 1999).

The second major factor that underlies the opponent activities of Nkx6.1 and Nkx6.2 in V1 interneuron specification appears to be a difference in the potency with

which the two Nkx6 proteins repress a common set of target genes. This view is supported by several observations. Nkx6.1 completely represses Nkx6.2, whereas Nkx6.2 exerts an incomplete negative regulation of its own expression in p1 domain progenitors. Thus, Nkx6.1 is evidently a better repressor of Nkx6.2 than is Nkx6.2 itself. Similarly, Nkx6.2 is coexpressed with Dbx2 in p1 domain progenitors, whereas Nkx6.1 excludes Dbx2 from p2 domain progenitors, indicating that Nkx6.1 also is a more effective repressor of Dbx2 expression than is Nkx6.2. Consistent with this view, Nkx6.2 fails to repress Dbx2 expression completely from ventral progenitors in *Nkx6.1* mutants. The fact that Nkx6.2 is only a weak repressor of Dbx2 is critical for the formation of the p1 domain since the maintained expression of Dbx2 in these cells ensures the exclusion of Nkx6.1 expression (Briscoe et al., 2000).

Our results do not resolve why Nkx6.2 is a weaker repressor than Nkx6.1 in vivo. Differences in the primary structure of Nkx6.2 and Nkx6.1 (Cai et al., 1999; Muhr et al., 2001) could result in an intrinsically lower repressor activity of Nkx6.2, when compared with that of Nkx6.1. But our findings are also consistent with the possibility that the two Nkx6 proteins have inherently similar repressor activities, and that the Nkx6.2 protein is merely expressed at a lower level. Indeed within p1 progenitors, the level of Nkx6.2 expression is clearly subject to tight regulation, with significant consequences for neuronal specification. The selective expression of Nkx6.2 in p1 progenitors, coupled with its weak negative autoregulatory activity, ensures a level of Nkx6 activity that is low enough to permit Dbx2 expression but is still sufficient to repress Dbx1 expression, thus promoting the generation of V1 neurons.

Our findings therefore reveal that a gradient of extracellular Shh signaling is translated intracellularly into stepwise differences in the level of Nkx6 activity along the ventral-to-dorsal axis of the neural tube. Moreover, the different Nkx6 protein activity levels within ventral progenitor cells are a critical determinant of ventral neuronal fate. Cells that express low or negligible levels of Nkx6 activity (p0 progenitors) are directed to a V0 neuronal fate, cells that express an intermediate Nkx6 activity level (p1 progenitors) are directed to a V1 fate, and cells that express a high Nkx6 activity level (pMN and p2 progenitors) are directed to a motor neuron or V2 fate (Figure 8).

Nkx6 Repressor Function and Neuronal Patterning by Derepression

The finding that many progenitor homeodomain proteins exert mutual-cross repressive interactions has led to a model of spinal neuronal patterning based on transcriptional derepression (Muhr et al., 2001). Similar crossrepressive interactions may establish regional progenitor domains in more rostral regions of the developing CNS (Toresson et al., 2000; Yun et al., 2001). A premise of this model is that transcriptional repression is exerted at two sequential steps in neurogenesis. One repressive step operates at the level of the progenitor homeodomain proteins themselves, but a second repressive step is exerted on neuronal subtype determinant factors that have a downstream role in directing neuronal subtype fates (Briscoe et al., 2000; Muhr et al., 2001).

Our analysis of *Nkx6* compound mutant mice provides direct support for this two-step repression model, and in addition indicates that progenitor homeodomain proteins and neuronal subtype determinants differ in their sensitivity to repression by the same class II protein. Normally, the functions of *Dbx1* and *Evx1* are required sequentially during the generation of V0 neurons (Pierani et al., 2001; Moran-Rivard et al., 2001). In *Nkx6.1*$^{-/-}$; *Nkx6.2*$^{+/ltz}$ mutants, however, the generation of Evx1/2^{+} V0 neurons occurs in the absence of expression of Dbx1 by neural progenitor cells. Dbx1 expression is therefore dispensable for V0 neuron generation under conditions of reduced *Nkx6* gene dosage. From these results, we infer that the net level of Nkx6 protein activity in ventral progenitor cells is still above threshold for repression of Dbx1 expression, but is below the level required for repression of Evx1 expression. These data therefore support the idea that Nkx6 proteins normally inhibit V0 neuronal fate by repressing the class I progenitor homeodomain protein Dbx1, and independently by repressing expression of the V0 neuronal subtype determinant Evx1.

A differential sensitivity of progenitor homeodomain proteins and neural subtype determinants to repression appears therefore to underlie the dissociation of progenitor cell identity and neuronal fate observed in *Nkx6* mutants. Such two-tiered repression is, in principle, necessary to specify neuronal fate through transcriptional derepression. In the case of Nkx6.1, for example, repression of Dbx1 and Dbx2 (and possible other unidentified repressors) should be sufficient to derepress motor neuron subtype determinants such as MNR2 and Lim3 in pMN progenitors. But, unless Nkx6.1 also represses the expression of V0 determinants, Evx1 expression would also be initiated in differentiating motor neurons, resulting in a hybrid neuronal phenotype. Indeed, under conditions in which *Nkx6* gene dosage is reduced or eliminated, some of the neurons generated from the position of the pMN domain do transiently express a hybrid motor neuron/V0 neuron phenotype.

The derepression model also invokes the idea that a major role of Nkx6 class proteins is to exclude the expression of Dbx2 and other proteins that inhibit motor neuron generation. This view offers a potential explanation of why a few residual motor neurons are generated in *Nkx6* double mutants. We find that in the absence of *Nkx6* gene function, residual motor neurons are generated only at early developmental stages, suggesting that progenitor cells within the position of the pMN domain have committed to a motor neuron fate prior to the onset of the deregulated ventral expression of Dbx2 and other motor neuron repressors. We note that a third *Nkx6*-like gene exists in the mouse, but this gene is not expressed in the spinal cord of wild-type or *Nkx6* mutant embryos (E. Anderson and J.E., unpublished data), and thus its activity appears not to account for the residual motor neurons generated in *Nkx6* double mutants. Importantly, the detection of residual motor neurons in *Nkx6* double mutants also provides evidence that Nkx6 proteins do not have essential functions as transcriptional activators during motor neuron specification, further supporting their critical role as repressors.

Finally, the present studies and earlier work on neurogenesis in the ventral spinal cord (Ericson et al., 1996;

Thaler et al., 1999; Arber et al., 1999; Sander et al., 2000) have provided evidence that newly generated neurons can sometimes express mixed molecular identities. These observations raise the possibility that repressive interactions that select or consolidate individual neuronal identities are not restricted to progenitor cells. Consistent with this view, Evx1 is required to establish V0 and repress V1 neuronal identity through an action in post-mitotic neurons (Moran-Rivard et al., 2001), although it remains unclear whether Evx1 itself functions in this context as an activator or repressor. Similarly, the homeodomain protein HB9 has been implicated in the consolidation of motor neuron identity, through repression of V2 neuronal subtype genes (Arber et al., 1999; Thaler et al., 1999). HB9 possesses an eh-1 Gro/TLE recruitment domain (Muhr et al., 2001), suggesting that HB9 controls the identity of post-mitotic motor neurons through a direct action as a transcriptional repressor. The consolidation of neuronal subtype identity in the spinal cord may therefore depend on transcriptional repressive interactions within both progenitor cells and post-mitotic neurons.

Experimental Procedures

Generation of *Nkx6.2* Mutant Mice

Mouse *Nkx6.2* genomic clones were isolated from a 129/Ola mouse genomic library. A targeting construct was constructed by inserting a tau-lacZ/pGKneo cassette into a 5 kb 5′ HindIII-NcoI fragment and a 2.7 kb 3′ SphI-AccI fragment. The linearized targeting construct was electroporated into E14.1 (129/Ola) ES cells. Cells were selected with G418 and screened by Southern blot analysis using a 200 bp 3′ AccI fragment, which detected a 6 kb wild-type band and a 2.9 kb mutant band. Recombinant clones were injected into C57BL/6J blastocysts to generate two chimeric founders, both of which transmitted the mutant allele. Mice homozygous for the mutant alleles were born at Mendelian frequency and survived through adulthood. All experiments involved mice maintained on a C57BL/6 background. The generation and genotyping of *Nkx6.1* mutant mice have been described previously (Sander et al., 2000). Compound *Nkx6* mutant mice were obtained by crossing *Nkx6.2*$^{+/tlz}$; *Nkx6.1*$^{+/-}$ double heterozygous mice. Genotyping was performed using Southern blot analysis.

Chick In Ovo Electroporation

Mouse *Nkx6.2* was isolated by PCR (Komuro et al., 1993) and chick *Nkx6.2* from a chick spinal cord library (Basler et al., 1993) using mouse *Nkx6.1* and *Nkx6.2* as probes. cDNAs encoding full-length mouse and chick *Nkx6.2* were inserted into a RCASBP(B) retroviral vector and electroporated into the neural tube of stage HH (Hamburger and Hamilton, 1953) 10–12 chick embryos (Briscoe et al., 2000). After 24–48 hr, embryos were fixed and processed for immunohistochemistry.

Immunohistochemistry and In Situ Hybridization Histochemistry

Immunohistochemical localization of proteins was performed as described (Yamada et al., 1993; Briscoe et al., 2000). Guinea-pig antisera were generated against an 11 amino acid N-terminal sequence of mouse Nkx6.2. Other antibodies used were rabbit anti-Lim3 (Ericson et al., 1997), mAb Hb9 (Tanabe et al., 1998), rabbit anti-Isl1/2 (Tsuchida et al., 1994), rabbit anti-Chx10 (Ericson et al., 1997), rabbit anti-En1 (Davis et al., 1991), mAb anti-Evx1/2, rabbit anti-Dbx1, rabbit anti-Dbx2 (Pierani et al., 1999), rabbit anti-Nkx6.1 (Jörgensen et al., 1999), mAb anti-Pax7 (Ericson et al., 1996), rabbit anti-βgal (Cappel), and goat anti-βgal (Biogeneseis). Images were collected on a Zeiss LSM510 confocal microscope. In situ hybridization was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993), using chick probes for *Dbx1*, *Dbx2* (Pierani et al., 1999), *Nkx6.1* (Briscoe et al., 2000), and *Nkx6.2*. A mouse probe for the 5′UTR of *Nkx6.2* comprised 346 bp upstream of the start ATG site. Whole-mount X-gal staining was performed as described (Mombaerts et al., 1996).

Acknowledgments

We thank S. Morton for antibodies and cDNA probes, B. Han for technical assistance, and J. Briscoe, J. Frisen, B. Novitch, and H. Wichterle for comments on the manuscript. We also thank K. MacArthur for help in its preparation. J.M. is supported by a fellowship from the Swedish Brain Foundation. J.E. is supported by the Royal Swedish Academy of Sciences, The Swedish Foundation for Strategic Research, The Swedish National Research Council, and the Karolinska Institute. M.S. is supported by the Deutsche Forschungsgemeinschaft (SFB 444). T.M.J. is supported by grants from NINDS. T.M.J. is an Investigator of the Howard Hughes Medical Institute. This paper is dedicated to Toshiya Yamada.

Received June 4, 2001; revised July 16, 2001.

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